# The role of two-component regulatory systems of *Streptococcus pyogenes* in virulence

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## Summary

The ability of *Streptococcus pyogenes* to adapt to different host environments represents a key feature during the infection process of this important human pathogen. Two-component regulatory systems (TCS) act as sensors and communicators of environmental changes, and their contribution to streptococcal pathogenesis has so far been poorly investigated. Therefore, the main objective of this thesis was the identification and characterization of these systems in *S. pyogenes* with respect to their potential role as contributors to streptococcal virulence.

Genome analysis of a sequenced *S. pyogenes* strain revealed the presence of thirteen putative TCS. Five streptococcal mutants with deletions in two-component systems were constructed and investigated for different virulence behaviour in comparison to the parental strain. The mutants deficient in the TCS FasCA, TCS09, TCS07, or TCS13 were less resistant to killing by human phagocytic cells, indicating a role in virulence. The two most attenuated deletion mutants, deficient in TCS07 and TCS13, respectively, were characterized in more detail.

The TCS07 mutant showed a loss of virulence in a mouse model of skin infection, expressed by survival of infected animals, development of smaller skin lesions, and a rapid clearing of the mutant from systemic organs and bloodstream in comparison to the wildtype strain. On the molecular level, the mutant lacked transcription of two major antiphagocytic virulence factors: M-protein and streptococcal inhibitor of complement (SIC). As expected, absence of M protein was accompanied by the loss of fibrinogen binding capability of the mutant. Additionally, the mutant failed to induce encapsulation with hyaluronic acid under *in vivo* conditions as it was observed for the wildtype strain.

The mutant deficient in TCS13 exhibited a reduced virulence in the investigated mouse skin model, as well, seen by survival of infected mice and clearing of bacteria from systemic organs and bloodstream when compared to the parental strain. Using a mouse model of intraperitoneal infection, the enhanced killing of this mutant within murine polymorphonuclear leukocytes (PMNs) could be demonstrated. Molecular analysis showed a reduced ability of this mutant to bind fibrinogen, which was found to be due to a release of the N-terminal part of the streptococcal M protein from its surface. Additionally, as with the TCS07 deletion strain, *in vivo*-induction of hyaluronic acid encapsulation did not occur in the TCS13 mutant. Thus, it was demonstrated that the two-component systems TCS07 and TCS13 of *S. pyogenes* are involved in regulation and posttranslational alterations of major streptococcal virulence factors and are required for full virulence of *S. pyogenes in vivo*.

# 1. Introduction

*Streptococcus pyogenes* (Lancefield Group A streptococcus, GAS) is a gram-positive, extracellular human pathogen. The name already describes its characteristic appearance of long chains (Fig. 1A): 'streptos' is the Greek word for "twisted"; 'coccus' means "berry". From the metabolic side, *S. pyogenes* belongs to the group of homofermentative lactic acid bacteria, generating this acid as main product. Furthermore, it is non-spore-forming, immotile, aerotolerant, and catalase-negative. A characteristic feature of *S. pyogenes* is the ability to lyse erythrocytes, a phenomenon called haemolysis. When grown on blood agar plates, this results in a clear zone surrounding growing colonies, also referred to as β-haemolysis (Fig. 1B). This β-haemolysis of *S. pyogenes* can clearly be distinguished from the α-haemolysis produced by *S. pneumoniae*, which induces colony-surrounding greenish zones.

**A**

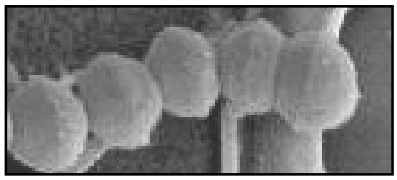


Figure 1. (A) Scanning electronmicroscopic photograph of a typical chain of *S. pyogenes* (by M. Rohde). (B) Clear β-haemolysic zones surrounding colonies of *S. pyogenes* on blood agar plate.

*S. pyogenes* is an exclusive human pathogen colonizing the throat and skin. There it can cause infections with a wide range of severities, ranging most commonly superficial infections such as pharyngitis, scarlet fever, or impetigo (47), to deep and life-threatening infections, such as bacteraemia and cellulitis, streptococcal toxic shock syndrome (STSS), and necrotizing fasciitis (56). Nonsuppurative autoimmune sequelae, such as rheumatic fever (RF) and glomerulonephritis can also develop following infections with *S. pyogenes* (47, 206) and are of major concern in developing countries. Annually, over 10 million GAS infections occur, most of them are non-severe throat and skin infections (press release, WHO 1999). However, since the mid-1980s an unexplained, worldwide resurgence of severe streptococcal infections has been observed, including systemic diseases, as well as outbreaks of STSS (9, 42, 208). These incidences, especially STSS, are associated with a high mortality rate of 30 - 60% since

there is no true therapeutic remedy (201). In developing countries, rheumatic fever is a major cause of cardiovascular mortality and remains an endemic disease; from 100000 school-aged children, annually 100 - 200 are affected by this streptococcal sequelae (164).

Regarding the treatment of streptococcal infections, penicillin is still the antibiotic of choice, and surprisingly no resistant strains have been reported up to now (96, 138). Nevertheless, failure to eradicate infection after penicillin-treatment has been reported in up to 25% of GAS pharyngitis (110, 173), suggesting that this pathogen has developed additional mechanisms to circumvent its effects. It has been proposed that intracellular survival of bacteria, as well as the degradation of this antibiotic by β-lactamase-producing coexisting commensals might be a reason (176, 194).

The ability of *S. pyogenes* to sense and respond to environmental signals is a key component to streptococcal pathogenicity. During infection, streptococci gain access to different compartments of the host where they propagate and persist. Therefore, *S. pyogenes* elaborated a broad range of virulence functions, which contribute to colonization or invasion of the epithelium, evasion of the host immune system, and dissemination from the local infection site of infection in order to cause more severe disease (47). These virulence functions are regulated by complex and overlapping regulatory circuits, which confer *S. pyogenes* a great flexibility to express different sets of genes at various host sites (47, 121). So far, only few of the regulatory systems of *S. pyogenes* have been identified and characterized. Therefore, the main objective of this study was to gain further insights into the underlying mechanisms of streptococcal virulence gene regulation with the focus on two-component regulatory systems (TCS), which contribute to signal perception and adaptation to environmental signals (204). Additionally, an enhanced understanding of the role of these systems in streptococcal virulence at specific host sites could provide crucial information for the identification of new targets for therapeutic intervention.

### 1.1. Virulence factors of *S. pyogenes*

Upon entering the host, several steps are crucial for *S. pyogenes* to establish an infection. The first step consists of the colonization of host surfaces, mediated by bacterial adhesins binding to the epithelium of the human respiratory mucosa or skin. Simultaneously, *S. pyogenes* needs to circumvent the host defence mechanisms, which includes (i) the avoidance of phagocytic uptake, (ii) survival within phagocytic cells, and (iii) degradation of immunoglobulins of the acquired immune system. All these processes are carried out by regulated virulence factors that enable the successful colonization and survival of *S. pyogenes.*

### 1.1.1. Adherence and colonization factors of *S. pyogenes*

Adhesion to host epithelial cells is the initial step for bacterial colonization and it protects *S. pyogenes* from being cleared by flow mechanisms of the mucosa and saliva (47). A large number of proteins involved in bacterial adhesion to host cells via the interaction with extracellular matrix proteins such as vitronectin, collagen, and laminin have been described, and the bacterial determinants binding either fibronectin or fibrinogen are the best characterized ones (107). Since *S. pyogenes* has a variety of adhesins, it is believed that the differential expression helps to colonize different niches of the host (216).

#### 1.1.1.1. Fibronectin binding proteins

Fibronectin is a glycoprotein that is present in plasma, as well as in the extracellular matrix of epithelial cells (37). Several proteins expressed by *S. pyogenes* have the ability to bind fibronectin, such as M1 and M3 protein (46, 189; see section 1.1.2.2.), Streptococcal fibronectin binding protein I (SfbI) (209) and its homologue protein F1 (85), as well as PFBP (185), SOF (44, 119;), and the glyceraldehyde phosphate dehydrogenase (165). All of these proteins are surface-associated and most of them contain an LPXTG-motif, linking them covalently to the cell wall (161). Additionally, the fibronectin-binding region is in some of these proteins conserved and has homologies to fibronectin-binding repeats of other bacteria.
Some fibronectin-binding proteins, such as SfbI and M1 protein can act as invasins and mediate internalization of *S. pyogenes* within epithelial cells (46, 157). The significance of this internalization process during streptococcal infection has been discussed controversially and the possibility that invading host cells can be used by *S. pyogenes* to acquire protection against antibiotic treatment has been hypothesized (176).

#### 1.1.1.2. Fibrinogen binding proteins

Fibrinogen is a glycoprotein present in large amounts in plasma, where it is a mediator of the blot-clotting cascade and wound healing (37). Although a number of streptococcal fibronectin-binding proteins can also bind fibrinogen (e.g. FBP54 (43)), the M protein seems to be the major determinant of *S. pyogenes* involved in the fibrinogen-binding activity (37). Functionally, the binding of fibrinogen to the streptococcal surface renders the bacterium resistant to uptake by host phagocytic cells (48, 183, 227). Thus, Whitnack and Beachey (227) have proposed that fibrinogen-acquisition via the M protein impedes the access of complement proteins to streptococcal cell wall structures, therefore protecting *S. pyogenes* against opsonization by complement. Additionally, the binding of fibrinogen leads to the

acquisition and activation of host plasminogen, which in turn is cleaved to plasmin by the streptococcal streptokinase (see section 1.1.3.3.), and might enhance bacterial invasion or movement of streptococci through tissue barriers (25, 38).

### 1.1.2. Antiphagocytic factors

The ability of *Streptococcus pyogenes* to survive and grow in whole human blood has long been known and is one of the most prominent features of this pathogen (126). The molecular basis of this resistance to phagocytosis is the expression of several factors important to circumvent the innate and adaptive host immune system. A number of host immune cells are important for the elimination of bacteria, above all macrophages, representing the first line of defence at early time-points of infection, and polymorphonuclear leukocytes (PMNs), which infiltrate infected tissues to efficiently kill invading streptococci (78, 220). The host complement system plays an additional essential role in host defence against streptococci and in the inflammatory process. Many complement proteins are involved in opsonization of microorganisms, rendering them susceptible to phagocytosis, killing and elimination (140). The major streptococcal factors responsible for resistance against uptake and killing by host phagocytic cells will be described in this chapter.

#### 1.1.2.1. Hyaluronic acid capsule

Hyaluronic acid capsule is a surface-located polysaccharide, which is composed of repeated units of glucuronic acid and N-acetylglucosamine (207). *S. pyogenes* strains expressing large amounts of capsule have a mucoid phenotype on culture plates, and this high expression has been associated with invasive streptococcal infections (108). Recently, Dinkla *et al*., (51) identified the role of the hyaluronic acid capsule in binding of streptococci to human collagen. The main contribution of the capsule to virulence, however, seems to be the involvement in rendering GAS resistant to killing by human blood leukocytes. Mucoid strains are highly resistant to phagocytosis, whereas loss of the capsule by a mutation within the capsule gene cluster (*has* operon) or by enzymatic treatment results in a higher susceptibility to phagocytosis *in vitro* (48, 70). Medina *et al.,* (152) proposed that the capsule is upregulated during intracellular location of *S. pyogenes* within PMNs, since these bacteria underwent a phenotypic switching from non-encapsulated to mucoid. Therefore, the upregulation of hyaluronic acid capsule might contribute to the ability of *S. pyogenes* to survive after phagocytosis. The role of the capsule in *in vivo* models of infection has also been shown, and a number of studies have reported encapsulated Group A streptococci to exhibit an enhanced

virulence (8, 104, 152, 223, 224).

### 1.1.2.2. M protein

The M protein is the best-characterized streptococcal virulence factor, and used for the differentiation of Group A streptococcal serotypes (125). It is covalently anchored to the bacterial surface by its C-terminus and is composed of four repeat blocks named A-D, all differing in size and sequence (95). The A-repeats are a hypervariable part of the protein, followed by variable B repeats and conserved C repeats. The D repeats are highly conserved and the proline-/glycine-rich region is with high probability located within the peptidoglycan layers (68) (Fig. 2). Due to a repeated periodicity of nonpolar amino acids, the M protein has an α-helical, coiled-coiled structure like mammalian tropomyosins, and appears as fibrils on the surface of Group A streptococci (172). During infection, antibodies against the N-terminal part of this protein are rapidly generated, leading to protection against Group A streptococcal infections of the same serotype (19). Consequently, the N-terminal part of the M protein is exposed to high selection pressure and undergoes frequent size variations, especially by insertion and deletions within the variable repeat regions (68).

The contribution of the M protein to bacterial virulence is diverse and ranges from adhesion to and invasion into host cells (29, 46), to tissue penetration (16), and antiphagocytic activity toward the innate and adaptive immune response (86, 98). The antiphagocytic effect of the M protein is mainly due to its ability to bind fibrinogen (see section 1.1.1.2.), as well as host complement regulatory protein factor H, leading to an inhibition of complement function (98). M protein has been found to be under the regulatory control of Mga (multiple regulator of GAS, 1.3.2.1.), which is a single regulator controlling the transcription of several virulence-associated genes (121). Since the M protein leads to the generation of protective antibodies against streptococcal infections (19), development of vaccine prototypes based on this protein are under investigation (49).

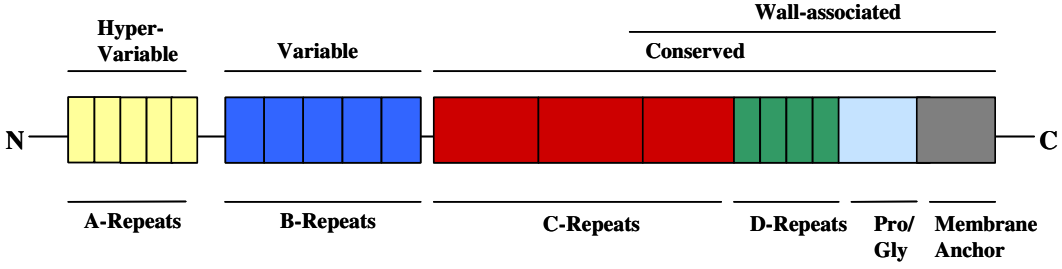



Figure 2. Schematic illustration of the structure of the streptococcal M-protein (based on Fischetti, 1989).

#### 1.1.2.3. Streptococcal inhibitor of complement-mediated lysis (SIC)

Protein SIC, originally described by Åkesson *et al.* (2), is a secreted protein that is predominantly present in *S. pyogenes* strains of the M1 serotype. This molecule exhibits a great polymorphism, higher than for any other streptococcal protein so far investigated (205), with almost 300 variants already identified (92). Such a high level of polymorphism is usually found in genes encoding for proteins that interact with the host environment (158). The function of SIC lies within its ability to bind regulators of the complement system, such as the human plasma proteins clusterin and HRG (histidine-rich glycoprotein) (2). This interaction leads to an inhibition of complement-mediated lysis of erythrocytes by incorporation of SIC into complement complexes (2). Additionally, SIC has an inhibitory effect towards two antibacterial peptides found in human neutrophils, α-defensin and LL-37 (74), as well as towards lysozyme and secretory leukocyte proteinase inhibitor (SLPI), which are components of the mucosal innate immune response (65). Hoe and colleagues (93), on the other hand, were able to show that the interaction of internalized SIC with the PMN component ezrin altered cellular processes critical for efficient bacterial internalization and killing. Therefore, in addition to the modulation of complement leading to evasion of the immune system, SIC is also capable of preventing the bactericidal activity of a number of host proteins.

#### 1.1.2.4. C5a-peptidase

The streptococcal serine protease C5a peptidase (ScpA) was originally identified by Wexler *et al*. (225), who described its endopeptidase activity towards C5a, a 74-residue chemotactic protein of the complement (64). This streptococcal surface-bound peptidase was shown to remove 6 amino acids from the C terminus of C5a, rendering it unable to interact with PMN receptors and therefore inhibiting the recruitment of phagocytic cells to the infection site. In animal experiments, intraperitoneal infection of C5a peptidase lead to a delayed accumulation of PMNs in the peritoneal cavity of mice, stressing the relevance of this activity for streptococcal pathogenesis. Transcription of C5a peptidase is under control of Mga (36; see section 1.3.2.1.). Interestingly, C5a peptidase can also be regulated at the postranslational level through proteolytic cleavage by the cysteine protease SpeB (17; see section 1.1.2.5).

#### 1.1.2.5. Cysteine protease SpeB

SpeB can be found as a secreted, as well as a surface-bound cysteine protease (105), and belongs to the streptococcal superantigens, which are further discussed in section 1.1.3.2. The SpeB gene is highly conserved (231), and under regulatory control of Mga (174; see 1.3.2.1.),

Rgg/RopB (regulation of proteinase) (135; see section 1.3.2.3.) and Pel (pleotropic effect locus) (130; see section 1.3.2.4.). Its transcription is induced during early stationary growth phase, but downregulated in the presence of glucose (32). SpeB is secreted as partially inactive zymogen and becomes enzymatically active after autocatalysis under reducing conditions (28). Functionally, this protease targets many host molecules, such as human fibrin (58), the antibacterial peptide LL-37 (191), IgG, as well as IgA, IgM, IgD, and IgE. The cleavage of IgG occurs at a defined site in the hinge region (40) and leads to a reduced capacity of opsonizing IgGs to kill *S. pyogenes* in human blood (41). The degradation of the other immunoglobulin-types seems to take place in a non-specific manner (40). Therefore SpeB seems to contribute to the resistance of *S. pyogenes* to phagocytosis in the presence of opsonizing antibodies.

However, SpeB does not only target host factors, but also other streptococcal surface proteins, such as C5a peptidase (1.1.2.4) and the IgG-Fc receptor binding surface molecule, protein H (1). The M protein can also be a substrate for SpeB, leading to the release of two internal fibrinogen-binding fragments after proteolytic degradation (17). Raeder *et al*. (178) associated the cleavage of 24 amino acids of the M protein's N-terminus by SpeB with decreased IgG binding properties and postulated a protective role of this action against opsonizing antibodies specifically recognizing this part of the M protein. Therefore, SpeB allows *S. pyogenes* to posttranslationally alter the activity of a number of its own proteins, contributing to a faster adaptation to different niches of the host. Additionally, modulation of host proteins necessary for recognition and killing of bacteria improves the ability of *S. pyogenes* to survive within its host.

### 1.1.3. Other virulence factors

#### 1.1.3.1. Streptolysin O (SLO) and Streptolysin S (SLS)

SLO and SLS are streptococcal exotoxins with haemolytic activities. SLO is present in nearly all *S. pyogenes* strains and is active in its reduced state, but rapid inactivation occurs in the presence of oxygen (3). It has high binding affinity to cholesterol, resulting in disruption of the eukaryotic cell membrane and subsequent lysis of the target cell (3). SLS, on the other hand is an oxygen-stable protein that is produced by all strains and is responsible for the β-haemolytic activity seen on blood agar plates (195). The contribution of both streptolysins in streptococcal virulence using animal models of infections has been extensively demonstrated (20, 131).

#### 1.1.3.2. Streptococcal Pyrogenic Exotoxins (SPEs)

*S. pyogenes* produces pyrogenic exotoxins with high similarities to staphylococcal enterotoxins (146). The expression of SPEs is associated with scarlet fever, as well as with streptococcal toxic shock syndrome (STSS) (42, 159). The role of these superantigens in streptococcal virulence lies in their ability to strongly induce stimulation of T-cells, which in turn leads to the release of large amounts of inflammatory cytokines and capillary leakage (226). SpeA, B, and C are the best characterized streptococcal exotoxins and due to its antiphagocytic effects, SpeB is discussed in more detail in section 1.1.2.5. Whereas the gene encoding for SpeB is present in all strains of *S. pyogenes*, the presence of SpeA and SpeC is variable among different GAS strains since they are bacteriophage-encoded (79, 109).

#### 1.1.3.3. Streptokinase

Streptokinase of *S. pyogenes* is a secreted plasminogen-binding protein, which is found in most streptococcal isolates (101). Due to the activity of this streptococcal protein, plasminogen is activated by its conversion to plasmin (134). The expression of streptokinase has been associated with the pathogenesis of acute poststreptococcal glomerulonephritis (141) and its contribution to streptococcal virulence and tissue invasion has been proposed (133, 123).

### 1.2. Bacterial signal transduction by Two Component Systems (TCS)

In order to survive in diverse and changing environments, bacteria have developed efficient regulatory systems to sense specific signals of their environment and to adapt their gene expression accordingly. Most of these systems are two component systems that are sophisticated signalling systems found mainly in eubacteria and archae, but also in a few eukaryotic organisms, such as yeasts and plants (203). Environmental signals can be of various natures, such as temperature, osmolarity, pH, light, oxygen, or nutrients, and even bacteria-derived quorum-sensing molecules, allowing inter- and intra-species communication (87). The regulatory outcome elicited by these systems is diverse and can range from sporulation (122), chemotaxis (5), and nitrogen regulation (83) to competence for genetic transformation (171), synthesis of exoproteins (142), and resistance to chemotherapeutics (61). In bacteria, the number of TCS change depending of the bacterial species: there are none TCS present in *Mycoplasma genitalium* (82), about 30 in *B. subtilis* (118), while 31 systems have been described in *E. coli* (232), and 13 in *S. pneumoniae* (127). These TCS are composed of at least two proteins: a histidine kinase (HK), and a response regulator (RR).

The histidine kinase is in most cases a transmembrane protein with a conserved kinase core; it functions as sensor molecule. The response regulator contains a conserved regulatory domain and most commonly modulates the transcription of target genes. The prototypic structure of a bacterial TCS is depicted in Fig. 3.

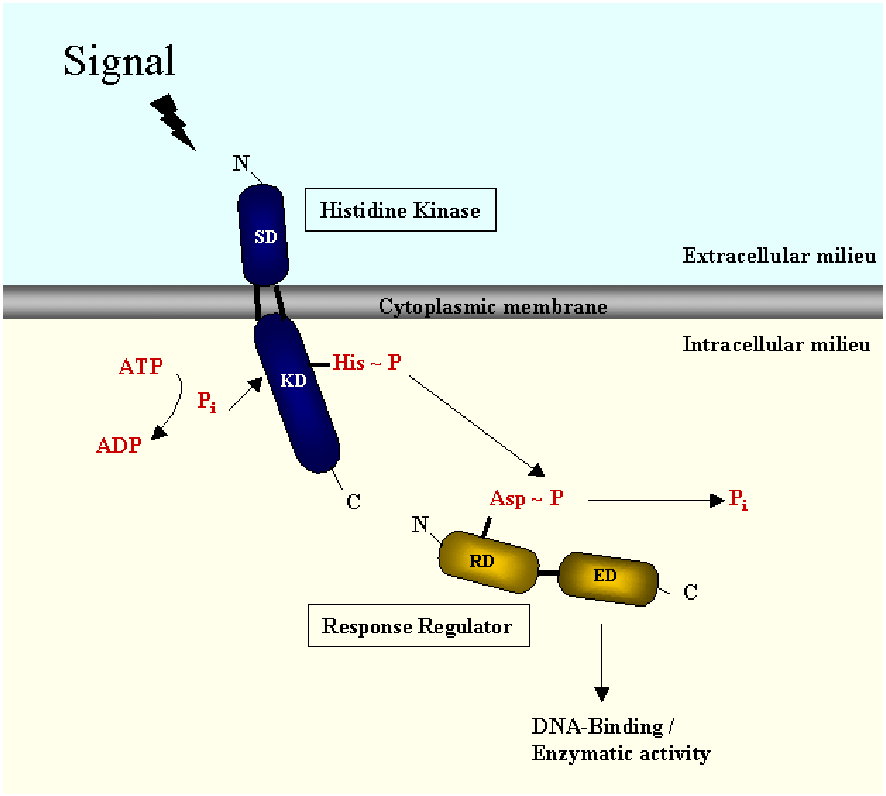



Figure 3. Schematic structure of a bacterial TCS: the histidine kinase as a transmembrane protein with its sensing- (SD) and kinase (KD) domain. The specific environmental signal induces the autophosphorylation of a histidine residue within the intracellularly located KD, the phosphoryl-group is transferred to an aspartate within the cognate response regulator, which harbours a regulatory- (RD), and an effector domain (ED). Phosphorylation of the response regulator can either result in DNA-binding, or enzymatic activity.

Generally, the N-terminal sensing domain of the HK is extracellularly located, or in the case of gram-negative bacteria, periplasmatically. The sensing domains of different TCS share little homologies since they respond to a variety of signals. Signals inducing activation of HKs are still unknown for most of the described TCS. The HKs can have two or multiple transmembrane helices; its C-terminus forms an independently folded domain that is located within the cytoplasm and comprises the kinase domain, which can bind ATP and has autokinase activity. Upon signal perception, it undergoes autophosphorylation at a highly

conserved histidine residue within the kinase core; a glycine-rich motif at the C-terminus is thought to bind the ATP needed for the phosphorylation activity. All phosphorylation steps require the presence of divalent cations, and it has been speculated that $Mg^{2+}$ is the most relevant cation *in vivo* (203). Although different HKs do not share high sequence homologies, so-called homology boxes, which are named due to characteristic conserved residues, can be distinguished. These boxes allow the classification into different histidine kinase subfamilies.
At least five of those homologous regions designated as H-, N, D-, F-, and G- boxes have been described; the first one (H) is directly involved in binding of the phosphoryl group, the others (N, D, F, and G) are involved in ATP-binding and kinase activity of the HK (204). Additionally, HKs contain an α-helical structure composed of a stretch of conserved residues beginning approximately 30 residues downstream of the phospho-accepting histidine, designated as X-region (82). The X-region seems to play an important structural role, as it was described for the HK EnvZ of *E. coli* (100). Some HKs have been shown to possess phosphatase activity towards their cognate RRs (203).
After signal perception and autophosphorylation of the histidine kinase, the phosphoryl group is transferred to the cognate response regulator (RR), which serves as catalyst for this transfer. RRs usually consist of two domains: a conserved N-terminal receiver domain, and a variable C-terminal effector domain. The phospho-accepting residue of the RR is a conserved aspartate present in the receiver domain. After phosphorylation, a high-energy acyl phosphate is generated and presumably used as energy source for the conformational change taking place on the RR (203). The lifetime of these phospho-aspartates can range from seconds to hours (90, 106, 221), and it has also been shown that many RRs have autophosphatase activity, thereby decreasing the lifetime of the phosphoprotein (90, 228).
Although RRs lack homology boxes, the presence of highly conserved single residues has been described (204). These include two conserved aspartates located at the N-terminal end of the RR, which are thought to be involved in the coordination of the magnesium ions required for the phosphorylation process. An additional conserved aspartate functions as phospho-acceptor and, together with a conserved serine (also often a threonine) and lysine crucial for the stability of the conformational change elicited by the phosphorylation event, all five residues surround the active site of the regulatory protein. The majority of RRs are transcription factors often harbouring a helix-turn-helix (HTH) DNA-binding motif that can activate or repress transcription of target genes upon phosphorylation. Some RRs however, lack this domain; instead, their C-terminal domain can have enzymatic activities as it was

shown for CheB (196).

Due to homologies within the DNA-binding domain of response regulators, TCS have been grouped into four different families (204). These are: (A) The OmpR family, characterized by the *E. coli* osmotic pressure sensor/regulator OmpR and EnvZ (71); (B) The AraC family, which was named after AraC, the arabinose operon regulatory protein of *E. coli* (76); (C) The family LuxR, their members have homologue HTH motifs to LuxR of *Vibrio fischeri* which activates the bioluminescence operon (88); and finally (D), the recently named LytTR ("litter") family of response regulators (also known as "Agr"), named after LytT of *Bacillus subtilis*, and LytR of *Staphylococcus aureus*, which are involved in the regulation cell autolysis (162). Since these families have been described, a large number of further TCS have been identified with additional features and grouped into many newly established subfamilies, which are based on functional, as well as sequence similarities. However, most identified TCS belong to the OmpR family, and many of these systems have been shown to be involved in nutrient perception and metabolic adaptations (204).

### 1.3. Regulatory systems of *S. pyogenes*

The genome-sequencing project of *Streptococcus pyogenes* SF370 (66) has revealed the presence of 13 different ORFs with homology to two-component systems (Tab. 1, also see section 2.1.). Of all these systems, only the CsrRS-TCS has been characterized in great detail (63), two other TCS named Irr/Ihk (63) and FasBCAX (120) have been studied to a lesser degree. The TCS SalRK and SrtRK are sensors and regulators in the context of the biosynthesis of lantibiotics, which are antibacterial peptides produced by some gram-positive bacteria (111, 186).

Tab. 1: Overview of characterized streptococcal two-component systems and regulated proteins.

| **Two-component system** | **Target protein** | **Environmental Signal** | **Reference** |
|---|---|---|---|
| CsrRS (= CovRS) | HasA, Ska, SLS, SpeMF | $Mg^{2+}$ | 63, 129 |
| FasBCAX | Fbp54, Mrp, | unknown | 120 |
| Irr/Ihk | Unknown | PMN contact | 63, 219 |
| SrtRK | Streptin-associated proteins | unkown | 222 |
| SalRK | Salivaricin-associated proteins | Salivaricin | 214 |

Besides these 13 TCS, a number of "stand-alone" regulators are also distributed throughout the GAS genome and, as for streptococcal TCS, only few have been characterized or shown

to be involved in virulence (Tab. 2). The following section will give an overview on known regulators in *S. pyogenes*, as well as their impact on the expression of virulence factors.

Tab. 2: Overview of characterized streptococcal single regulators and regulated proteins.

| Single Regulator | Regulated proteins | Environmental Signal | Reference |
|---|---|---|---|
| Mga | M protein, C5a peptidase, SOF, SIC, Mga | Elevated $CO_2$, iron limitation, increased temperature | 168,198 |
| RofA | RofA, SfbI, SLS, SpeB, Mga | Temperature | 69, 199 |
| Nra | Cpa, SfbII, SpeB, SpeA, SLS, Mga | unknown | 175 |
| Rgg/RopB | M protein, C5a peptidase, HasA, SLO, SLS, Mga, FasBCA, Irr/Ihk, SalRK, CsrRS | Temperature | 33,135, |
| SagA/Pel | Ska, SpeB | unknown | 130 |
| LuxS | SLS, SpeB | Autoinducer AI-2 | 136 |

### 1.3.1. Streptococcal TCS

#### 1.3.1.1. Capsule synthesis regulator/sensor (CsrRS)

The two-component system CsrRS, also named CovRS for control of virulence genes (63) is by far the best characterized TCS in *S. pyogenes*. It belongs to the OmpR family of TCS and has been identified as a repressor of the capsule gene cluster (*has* region) in different GAS serotypes. The *has* operon is composed of three open reading frames, consisting of the has synthase (*hasA*), UDP-glucose-dehydrogenase (*hasB*), and pyrophosphorylase (*hasC*), all involved in the synthesis of hyaluronic acid. The response regulator CsrR has been shown to directly bind to a consensus element within the *has* promotor region (18, 129), repressing its transcription. This regulatory system critically influences virulence since the capsule is a major virulence factor contributing to the outcome of streptococcal infections (104; see section 1.1.2.1.). In addition to the regulation of hyaluronic acid capsule expression, CsrRS has also been associated with repression of streptokinase, SLS, and superantigens SpeM and F (63). Furthermore, genes essential for general stress responses seem to be affected by this regulatory system, since a CsrRS mutant was unable to grow at low pH, elevated temperature or osmolarity (50). Gene transcription profiling by microarray analysis of a CsrR mutant strain revealed that of the whole streptococcal genome, transcription of about 15% of the genes, including a number of virulence factors and regulatory systems such as TCS10 and

Irr/Ihk, are affected by this mutation (80). Therefore, it was proposed that CsrRS is a part of a more complex regulatory network in GAS (80). The contribution of CsrRS to bacterial virulence was evidenced by the more severe ulcer- and lesion development in a skin infection model observed after *in vivo* infection with a strain carrying a mutation in CsrR. The more severe phenotype of infection seemed to be due to the higher expression of capsule and SLS (60). Additionally, spontaneous mutation of the CsrRS locus, resulting in higher capsule expression was frequently observed (59). The CsrRS system is the only TCS in GAS for which a molecular environmental signal has been identified. Gryllos *et al*. (79) observed that addition of $Mg^{2+}$ to the growth medium resulted in repression of the capsule expression by up to 80 %, a phenomenon that did not occur in the mutant. However, since the phosphorylation events in TCS require the presence of divalent cations (203), the direct interaction of the sensor with the proposed stimuli still needs further investigation.

#### 1.3.1.2. FasBCA(X)

Unlike the prototypic TCS illustrated in Fig. 2, the FasBCAX system is composed of two histidine kinases and one response regulator, suggesting that diverse signals may be needed for elicitation of a response. This regulatory system belongs to the LytTR- or Agr-family of TCS, which has been associated with the regulation of virulence genes in *Staphylococcus aureus* (167). The main effector of this system named FasX has been identified as an untranslated regulatory RNA (120). Disruption of FasBCA resulted in a growth-phase-dependent reduced expression of the virulence factors streptokinase and SLS, as well as to a downregulation of the adhesion proteins FBP54 and MRP (120). Additionally, FasBCA expression seems to be influenced by the regulator Rgg (34; see 1.3.2.3). Little is known about the role of this TCS in streptococcal virulence. However, the high expression of the histidine kinase FasC observed during acute pharyngitis may suggest a putative role of this TCS during the infection process (218).

#### 1.3.1.3. Isp-adjacent RR/HK (Irr/Ihk)

The Irr/Ihk system (named TCS13 in the work presented here) was initially described by Federle *et al*. (63). They investigated the contribution of this system to the transcription of virulence genes encoding the M protein, C5a peptidase, SLO, SLS, hyaluronic acid capsule, and streptokinase by a mutagenesis approach, but did not find any significant differences between wildtype and mutant. Up to date, the streptococcal virulence factors or other genes under direct or indirect regulatory control of this system remain unknown. However, a high

expression of Irr/Ihk during acute pharyngitis has been reported (218). Furthermore, the activity of this regulatory system seems to be important for the mechanism by which intracellularly located *S. pyogenes* evade killing by human PMNs. This was shown by an upregulation of this system during the encounter with human PMNs and the failure of Irr/Ihk deficient mutants to survive within PMNs (219). Additionally, Irr/Ihk expression seems to be positively influenced by the TCS CsrRS (80).

#### 1.3.1.4. Lantibiotic-associated TCS

Lantibiotics are antimicrobial peptides produced by some gram-positive bacteria and are active against closely related species. Their name derived from the presence of the modified amino acid lanthionine. Lantibiotics are synthesized as propeptides and posttranslationally processed (84). The biosynthesis of lantibiotics is depending on the presence of an inducer molecule, which is sensed by the corresponding lantibiotic-associated HK, subsequent transcription is induced by the RR-activity.

Salivaricin is described as a lantibiotic produced by *Streptococcus salivarius*, but is however found in many *S. pyogenes* strains of different serotypes (214). The TCS SalRK lies within the salivaricin gene cluster that comprises six other genes involved in the production, processing and transport of this lantibiotic. In *S. pyogenes* strain SF370, the gene encoding the salivaricin precursor is transcriptionally inactive due to a deletion within the gene cluster (214). In a *Streptococcus salivarius* strain that harbours the complete salivaricin cluster, the involvement of SalRK in salivaricin synthesis, as well as its auto-regulation by external salivarcin concentrations has been demonstrated. Additionally, it was speculated that an additional, unknown regulator might be involved and the role of salivaricin as intra- and interspecies signalling molecule between *S. salivarius* and *S. pyogenes* has also been proposed (214).

The TCS SrtRK lies within the *srt*-gene cluster, which encompasses five other genes involved in streptin synthesis, transport, and in so far unknown functions. Similarly to the genes coding for the lantibiotic salivaricin, *S. pyogenes* strain SF370 harbours an incomplete *srt*-locus due to a deletion of streptin processing- and transport genes, therefore resulting in lack of the active lantibiotic (222). Although the gene for streptin (*srt*A) is widespread among *S. pyogenes* strains of many serotypes, only few of them are able to produce the active lantibiotics (222). Neither the role played by the SrtRK system in the biology of *S. pyogenes*, nor its environmental signal is known.

### 1.3.2. Regulation by "Stand-alone" regulators

In addition to the typical TCS, other regulatory systems which seem to function without a cognate histidine kinase have been described. These "stand-alone" regulators have been shown to regulate a number of virulence genes of *S. pyogenes*. Some of them are similar to response regulators of TCS and may in fact be associated with so far unidentified sensory elements; others are unrelated transcriptional regulators.

#### 1.3.2.1. Multiple gene regulator of GAS (Mga)

Of all GAS regulators, Mga is by far the best characterized. It was originally described by Simpson *et al.* (198) and named *vir*. Mga can be found in all GAS serotypes and contains sequence similarities with and features of response regulators, such as a phosphorylation acceptor motif (168). For this reason, it was proposed that Mga might represent the response regulator of a two-component regulatory system. However, the putative sensor protein that responds to environmental signals and activates Mga remains unidentified. Mga is auto-regulated and additionally influenced by elevated temperatures and $CO_2$-levels, as well as iron limitations (31, 147) and it positively regulates several GAS virulence factors, including M protein, C5a peptidase, serum opacity factor, and SIC protein (121). The Mga-regulated genes have been identified by direct Mga-binding studies to the corresponding promotor sequences (150), therefore this regulator seems to directly influence gene expression of these virulence factors, which are expressed during mid- to late exponential growth phase (149). *In vivo* transcriptional analysis using swabs from patients with acute pharyngitis revealed a high expression rate of Mga, underlining its role for the regulation of virulence factors active during *in vivo* infection (218).

#### 1.3.2.2. Regulator of F (RofA) and Negative regulator of GAS (Nra)

Due to the 62% sequence identity shared by RofA and Nra, they were grouped together in a family called RALP (RofA-like protein family of regulators) (121). Despite high similarity on the sequence level, they have different regulatory functions. The fibronectin-binding protein of GAS, SfbI/protein F, as well as RofA itself is under regulatory control of RofA, as shown by DNaseI protection assays (81). Furthermore, it has been suggested that RofA also regulates SLS, SpeB, and the regulator Mga (14). A reduced transcription of the *rofA* gene could be observed at reduced temperatures (199).

Similar to RofA, Nra seems to regulate expression of colonization factors such as Cpa, a collagen-binding protein, and another fibronectin-binding protein of *S. pyogenes*, SfbII (175).

Additionally, Nra can repress the expression of other virulence genes, including *speB, speA, sagA* (SLS), and *mga* (121).

#### 1.3.2.3. Rgg

Rgg, also known as RopB, was first identified as a regulator for SpeB expression (33). Microarray transcriptome analysis of an Rgg mutant in comparison with the wildtype strain has identified a large number of genes transcriptionally affected by this regulator. These include the genes encoding the M protein, C5a peptidase, hyaluronic acid synthetase, SLO and SLS, as well as a variety of known two-component-, and stand-alone regulators, such as Mga, SagA CsrRS, Irr/Ihk, SalRK, and FasBCA (34). Additionally, decreased temperature has been found to increase transcription of this regulator (199). Independently, an unusual utilization of nutrients such as arginine and serine instead of glucose, accompanied by an upregulation of enzymes needed for this utilization, was also observed in the mutant strain (35). The complexity of bacterial regulatory circuits can clearly be seen by the abundance of genes influenced by this single regulator. Genes under direct control of Rgg, however, have not yet been identified.

#### 1.3.2.4. Other Regulators

RocA (Regulator of csr) is a membrane-associated regulator without known DNA binding motif, which lead to the assumption that it is not a regulator on the transcriptional level. However, deletion of this gene leads to an elevated level of CsrR transcript, resulting in a mucoid phenotype of the mutant (22), and therefore implying a potential role of this regulator in streptococcal virulence.

LuxS is a regulator known for its role in quorum-sensing and cell density-dependent gene expression in other bacteria, such as *S. mutans*, where it responds to a secreted pheromone named autoinducer-2 (154). Deletion of the homologue in *S. pyogenes* has been shown to result in a media-dependent growth defect, as well as an increased expression of SLS, and a lower secretion level of the SpeB precursor (136). Functionally, LuxS might be involved in invasion of epithelial cells, since the lack of this regulator increased the internalization rate of *S. pyogenes* (143).

Deletion of the *sagA* locus, encoding SLS, has also been shown to influence the expression of several virulence genes. Li *et al.* (130), who named this locus *pel* for pleiotropic effect locus found that this deletion reduced the expression levels of streptokinase and SpeB. In addition, SLS mutant strains have been shown to be more attenuated than the wildtype strain in a

mouse model of GAS infection (20). Whether the observed reduced virulence was due to lack of this important virulence factor in the SLS mutant, or to the missing regulatory activity of SLS on other virulence genes remains to be elucidated.

### 1.4. Objectives of this work

The extend to which pathogenic bacteria are able to percept and adapt to changes within their host is an important attribute with respect to bacterial virulence, and it greatly influences the outcome of infection. Most Group A streptococcal two-component systems are so far poorly investigated and little is known about their role in gene regulation and virulence.

Within the scope of this dissertation, the role of two-component systems in streptococcal virulence was to be investigated. Therefore, the objectives of this work were the identification of such systems involved in streptococcal virulence, as well as the elucidation of underlying mechanism responsible for this involvement. This was to be achieved by (i) the construction of a number of stable TCS knock-out mutants, (ii) a closer characterization of the mutants' virulent behaviour in a mouse model of infection, and (iii) identification of virulence factors regulated by the corresponding TCS.

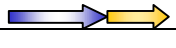
# 2. Results

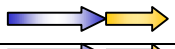
The first part of this section addresses two-component systems in strain KTL3, the generation of deletion mutants in 5 of these systems, and their investigation as contributors to bacterial virulence. The second part focuses on the closer characterization of two of these systems with corresponding mutants, which showed impairment in their virulence behaviour.

## 2.1. Two-component systems in *S. pyogenes* strain KTL3

Screening of the annotated genome of *S. pyogenes* strain SF370 (66) for putative two component systems revealed a total number of 13 TCS present in this strain. In most cases, the genes for TCS lie adjacent to each other on the bacterial chromosome and are transcribed as an operon. The systems present in SF370 were named according to their order in this genome, except already characterized ones, for which given names were retained. Additionally, some systems were named due to their homology to other TCS. Homology searches by BLAST and comparison of the molecular designs of these systems allowed their classification into the described four families (LytTR, OmpR, AraC, LuxR; see section 1.2.), except for TCS07. This system shows the closest homology to the LytTR (AgrA) family, however can be grouped into a newly described family called CitAB (156).

Tab. 3: Two component systems present in *S. pyogenes* strain SF370.

| No. | Name | ORF designation | Accession number | TCS-family | Gene organization | Reference |
|---|---|---|---|---|---|---|
| 1 | FasBCA | Spy 0243/0244/0245 | AE006491 | LytTR |  | 120 |
| 2 | CsrRS (= CovRS) | Spy 0336*/0337 | AE006498 | OmpR |  | 129 |
| 3 | VicRK | Spy 0528/0529 | AE006510 AJ012051 | OmpR |  | |
| 4 | TCS04 | Spy 0874/0875 | AE006537 | OmpR |  | |
| 5 | TCS05 | Spy 1061/1022 | AE006550 | AraC |  | |
| 6 | SrtRK | Spy 1081/1082 | AE006552 | OmpR |  | 116 |
| 7 | TCS07 (DpiAB) | Spy 1106/1107 | AE006553 | CitAB |  | |
| 8 | TCS08 | Spy 1237/1236 | AE006563 | OmpR |  | |
| 9 | TCS09 | Spy 1555**/1556 | AE006558 | AraC |  | |

| | | | | | | |
|---|---|---|---|---|---|---|
| 10 | TCS10 | Spy 1587/1588 | AE006590 | AraC |  | |
| 11 | TCS11 | Spy 1621/1622 | AE006593 | LuxR | | |
| 12 | SalRK | Spy 1908/1909** | AE006616 | LuxR |  | 186 |
| 13 | Irr/Ihk (TCS13) | Spy 2026/2027 | AE006624 | OmpR |  | 63 |

Histidine kinases are displayed in blue, response regulators in yellow. * This gene has not been annotated and therefore not named in the SF370 genome.** This gene has not been annotated and named due to a frameshift mutation in the SF370 genome.

For identification of TCS involved in streptococcal virulence, *S. pyogenes* strain KTL3 (179), which had been used before in an established murine skin- infection model (213) was chosen for disruption of these systems. Since the number of TCS systems, especially those associated with the expression of lantibiotics (see section 1.3.1.4.) may vary from strain to strain, the total number of TCS present in strain KTL3 had to be determined. Oligonucleotides were chosen on the basis of the SF370 sequence, and KTL3 was screened for the presence of TCS by PCR. Figure 4A shows the amplified DNA fragments of the corresponding TCS in strain SF370, Fig. 4B displays the TCS-fragments of KTL3, revealing that all systems are present in this particular strain.

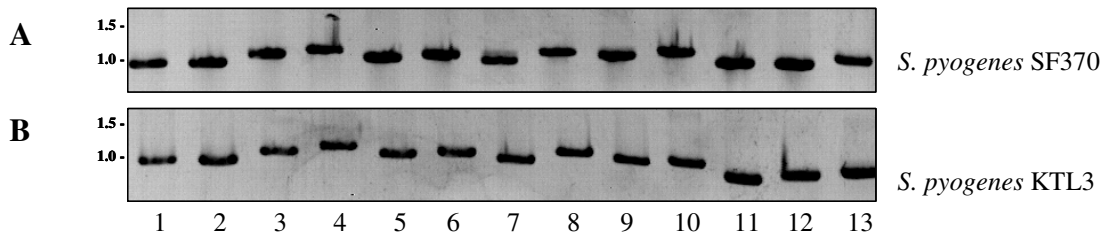



Figure 4: Agarosegel of the PCR analysis showing TCS present in strains SF370 (A) and KTL3 (B). Numbers refer to the corresponding ones in Tab. 3, representing all 13 TCS. Fragment sizes are indicated as kb on the left.

## 2.2. Generation of TCS-deletion mutants in *S. pyogenes* strain KTL3

For assessing a possible role of TCS in streptococcal virulence, deletion mutants for FasBCA, TCS07, TCS09, TCS11, and TCS13 were generated in strain KTL3. These particular systems were chosen for the following reasons. (i) The FasBCA system has already been shown to play a role in the regulation of virulence factors (120), and therefore a regulatory mutant deficient at this locus would allow further insight into the system's role in bacterial virulence. (ii) TCS13 has been chosen due to its chromosomal neighbourhood (2.4.2.1.), which is mainly composed of characterized virulence factors (Fig. 28 page 54). (iii) TCS 07 (recently named DpiAB by Banks *et al*., (12)) and 11 have been chosen based on their gene organization, in which the histidine kinase precedes the response regulator (Tab. 3). Most of the TCS involved in nutrient perception and metabolism belong to the OmpR family of TCS (see section 1.2.), which are typically for theses systems arranged in the opposite way (Tab. 3). Therefore, these systems were neglected for mutagenesis studies, except for TCS09.

### 2.2.1. Construction of plasmids used for electroporation

For disruption of the corresponding TCS in strain KTL3, a promotor-containing spectinomycin resistance cassette (145) was inserted into the first gene of the corresponding system, which was either the histidine kinase or the response regulator depending on its gene arrangement (Tab. 3). As exception, in the FasBCA system, which is composed of two histidine kinases and one response regulator (Tab. 3), the insertion was introduced within the second histidine kinase, therefore leaving the first kinase intact upon chromosomal integration. As result of the insertion, a frameshift mutation is introduced into the targeted gene and due to a transcriptional stop inserted at the 3'-end of the spectinomycin cassette, the second TCS gene will no longer be co-transcribed.

An overview of the plasmid construction, as well as following transformation- and incubations steps is given in Fig. 5 on page 23. Constructs for gene inactivation were partially obtained by overlap extension PCR (see section 5.6.3.3.). Three sets of primers were used for each construct and PCR products were amplified from the appropriate template DNA. The first set of oligonucleotides was used to amplify the homologous fragment 1, corresponding to the 5' end of the gene to be inactivated; the second set was used to amplify the spectinomycin cassette, and the third set was used to amplify the homologous fragment 2, corresponding to the 3' end of the gene to be inactivated. PCR-derived fragments 1 and the spectinomycin cassette were purified (5.6.4.), mixed in a ratio of 1:1, and reamplified in a second run with

the external primers. The resulting fragment was ligated into the pCR2.1 cloning vector resulting in pCR2.1-F1-spc, the same was performed with the homologous fragment 2, giving rise to pCR2.1-F2 (Fig. 5, step 1). Fragment 2 was then excised from the vector using the vector-harbouring restriction site *Xho*I in combination with the primer-harbouring site *Avr*II, and ligated into the *Avr*II/*Xho*I restricted pCR2.1-F1-Spc, resulting in pCR2.1-F1-Spc-F2 (Fig. 5, step 2 and 3).

For transformation of *S. pyogenes*, the thermosensitive shuttle vector pJRS233 (170) was used, which is a derivative of pG$^+$host4 (139). In *E. coli*, this vector can autonomously replicate at 37°C, but in *S. pyogenes* only at 30°C. Therefore, upon raise of temperature to 37°C under selective pressure, only chromosomal integrates of the plasmid are recovered in *S. pyogenes*. The above constructed F1-Spc-F2 fragment was excised from pCR2.1 using vector harbouring restriction sites *Hind*III or *Spe*I, in combination with the vector harbouring site *Xho*I, and ligated into the shuttle vector pJRS233, resulting in pJRS233-RR/HK::Spc (Fig. 5, step 4), which was then used for transformation of KTL3. All plasmids used for electroporation are listed in Tab. 4.

Tab. 4: Plasmids generated in this work used for electroporation of *S. pyogenes* strain KTL3

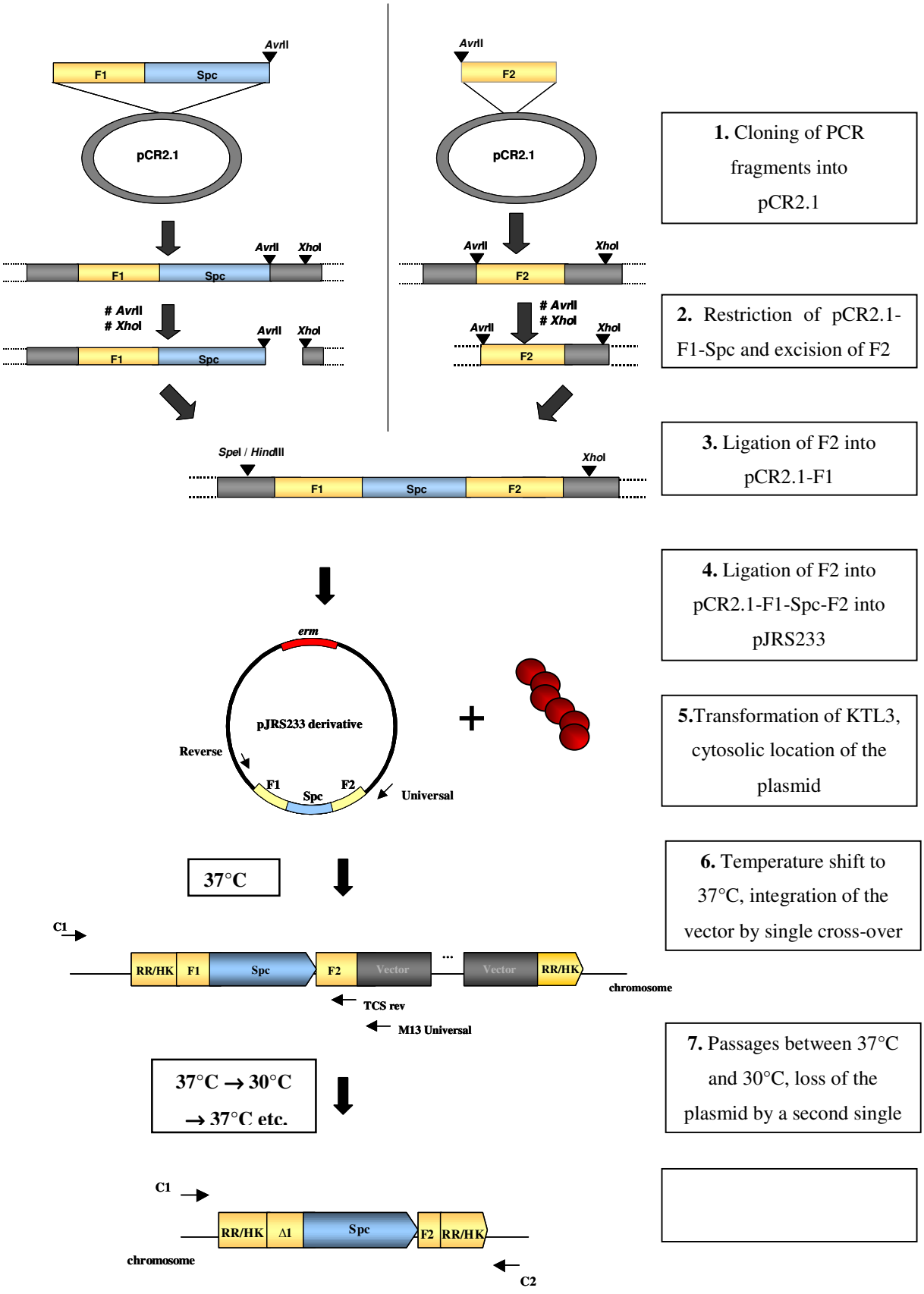
| **Plasmid** | **Selection** | **Cloned DNA-fragments*** |
|---|---|---|
| pJRS233_fasC::spc | Spc (80 µg/ml) | *fasC*(1): 211495-211946<br>aad9<br>*fasC*(2): 211995-212461 |
| pJRS233_hk07::spc | Spc(80 µg/ml) | *hk07*(1): 907423-906990<br>aad9<br>*hk07*(2): 90694-906485 |
| pJRS233_hk09::spc | Spc (80 µg/ml) | *hk09*(1): 12821138-1281712<br>aad9<br>*hk09*(2): 1281629-1281136 |
| pJRS233_hk11::spc | Spc (80 µg/ml) | *hk11*(1): 1347919-1344476<br>aad9<br>*hk11*(2):1344458-1344007 |
| pJRS233_irr::spc | Spc (80 µg/ml) | *irr*(1): 1691848-1691361<br>aad9<br>*irr*(2) : 1691323-1690860 |

*The fragment positions correspond to the GenBank accession numbers NC_03737 (*S. pyogenes* SF370, complete genome) and M69221 (*aad9*).

### 2.2.2. Electroporation of *S. pyogenes*

For electroporation of *S. pyogenes* strain KTL3, a method adapted from McLaughlin and Ferretti (151) was applied. Using the recommended TH-Broth with glycine however did not yield any transformants; only exchange of the medium to TSB with glycine resulted in successful transformation. Since transformation with at –70°C stored bacteria was inefficient, cells were freshly prepared before each transformation and resuspended in the electroporation buffer described by Caparon and Scott (30). Additionally, the suggested amount of DNA (1-2 µg) was raised to up to 8 µg, amounts below 5 µg did not yield any transformants.

After electroporation of *S. pyogenes* strain KTL3 with the pJRS233-derivatives (Tab. 4 and Fig. 5 step 5), the cultures were maintained in selective medium (0.5 µg/ml Erm) at 30°C for allowing autonomous replication of the plasmid. Verification of the presence of the plasmid was achieved by PCR using oligonucleotides M13 Universal and Reverse (for priming sites in the plasmid refer to Fig. 5B). Subsequently, clones were subjected to a temperature shift to 37°C, selecting for chromosomal integration of the plasmid into the corresponding TCS by a single cross-over event (Fig. 5 step 6). This genetic setup can only be reliably maintained under constant selective pressure, preventing reversion to the wildtype situation by homologous recombination within the identical copies of the internal gene fragment concomitant with the excision of the plasmid (Fig. 5 step 7). However, for animal infection experiments, occurring in the absence of selective antibiotics, stable, non-revertible double cross-over mutants were required. Therefore, clones were passaged between 30°C and 37°C (Fig. 5 step 7). The shifts back to 30°C permit the autonomous replication of the plasmid and favour the excision of the plasmid from the chromosome. Excision of the plasmid from the chromosome results either in reversion to the wildtype situation or in replacement of an internal gene fragment by the spectinomycin resistance cassette, depending on the exact position of homologous recombination. Transformants were selected on spectinomycin for this second genetic setup and repeatedly analyzed until complete loss of the plasmid and integration of the spectinomycin cassette into the genome was confirmed (Fig. 5 step 8). This transformants were resistant to spectinomycin and, due to the loss of the plasmid, sensitive to erythromycin.




**1.** Cloning of PCR fragments into pCR2.1

**2.** Restriction of pCR2.1-F1-Spc and excision of F2

**3.** Ligation of F2 into pCR2.1-F1

**4.** Ligation of F2 into pCR2.1-F1-Spc-F2 into pJRS233

**5.**Transformation of KTL3, cytosolic location of the plasmid

**6.** Temperature shift to 37°C, integration of the vector by single cross-over

**7.** Passages between 37°C and 30°C, loss of the plasmid by a second single

Figure 5: Schematic overview illustrating the construction of the pJRS233-derivatives used for transformation of KTL3 and the process of transformation and integration of the plasmid into the streptococcal chromosome, yielding stable insertion of the spectinomycin cassette into the targeted gene. Additionally, priming sites for oligonucleotides used for PCR analysis of KTL3 transformants are indicated. F1 and F2 correspond to the two internal fragments of the TCS; Spc refers to the spectinomycin resistance cassette, and RR/HK represent the chromosomal location of the targeted gene. Priming sites are indicated as arrows. (B) Priming sites of Universal and Reverse on the pJRS233-derivatives for step (i) described in 2.3.1; (C) Priming sites of oligonucleotides for verification of the chromosomal integration described in step (ii) of 2.3.1; (D) Final verification of the double-cross-over described in step (iii) of 2.3.1. For further descriptions refer to the text.

## 2.3. Verification of TCS deletion mutants

### 2.3.1. PCR analysis of KTL3 transformants

A fast screening of the mutants by PCR was achieved in three independent reactions. (i) Using vector-priming oligonucleotides M13 Universal and Reverse (Figure 5 B); a negative PCR result indicated the loss of the free form of the plasmid. (ii) Loss of the plasmid from the chromosome was confirmed by negative PCR using oligonucleotide M13 Universal in combination with a TCS-specific one (Fig. 5 C). (iii) The final verification of the double cross-over event and integration of the spectinomycin resistance cassette into the locus was obtained by using oligonucleotides C1 and C2, which prime outside of the site of recombination (Fig. 5 D). Due to the inserted resistance cassette, the resulting PCR products were larger when compared to the wildtype (Fig. 6).

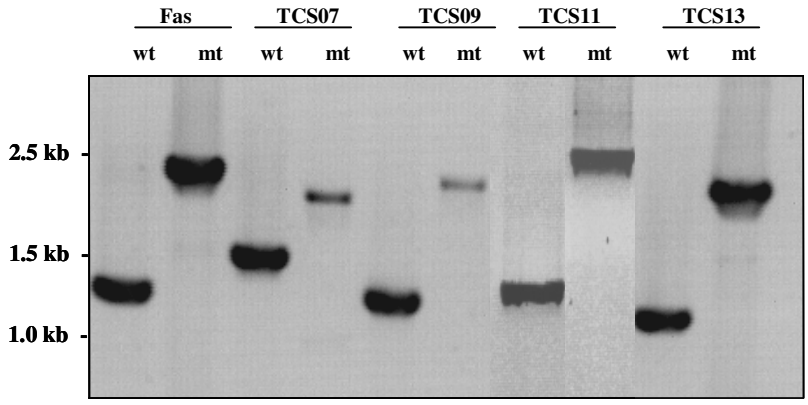



Figure 6: Verification of the double cross-over by PCR. Oligonucleotides C1 and C2 for the corresponding TCS were used, wt refers to the parental strain KTL3, mt stands for the knockout strains. Fragments amplified from the mutants are larger due to the insertion of the spectinomycin resistance cassette.

### 2.3.2. Sequence analysis of the integration site in the TCS mutants

Sequence analysis of the regulatory mutants was performed as final proof for verification of the correct integration of the spectinomycin cassette into the corresponding TCS loci. As seen in Fig. 7, *fasC* has the cassette integrated at position 326, corresponding to the glutamine at position 109 of the histidine kinase. HK07 has the cassette integrated after position 404, which corresponds to the lysine in position 134, RR09 after amino acid 88 (tyrosine), HK11 after amino acid 137 (alanine) and RR13 after amino acid 142, which is a glutamine. These results confirm the correct integration of the spectinomycin cassette into the corresponding TCS. Integration sites of the spectinomycin cassette determine the size and putative remaining activity of the residual mutant protein, i.e. the N-terminal part of the protein might be expressed up to this position in the mutant strain. All inactivated histidine kinase genes are disrupted upstream of the encoded crucial histidine residue, resulting in loss of the functional kinase domain. For RR09 and RR13, a residual protein will only cover the phosphorylation domain, but will lack the DNA-binding domain. The insertion leads to a frameshift mutation within this gene, resulting in a premature mRNA-termination due to the transcriptional stop present at the 3'-end of the spectinomycin cassette. Therefore, the second gene of the TCS will not be cotranscribed, as well as other genes located within the same operon.

```
FasC   300 GCTGTCTTAGGATTTAGCATTGGTCAACCCGGGCAATTGCCCGGATCGATTTTCGTTCGTGAATACATGTTAT
HK07   404 ATGGCGGTTCCCGTGTTTAGGAAAGTCCCCGGGCAATTGCCCGGATCGATTTTCGTTCGTGAATACATGTTAT
RR09   266 CGTTCTAGATGCGGTGTCAGGCTATCGCCCGGGCAATTGCCCGGATCGATTTTCGTTCGTGAATACATGTTAT
HK11   386 TAAAACAAGAGCGCAAACGAATTGCAACCCGGGCAATTGCCCGGATCGATTTTCGTTCGTGAATACATGTTAT
RR13   223 GAGACCGAACATAGCGTCTATTGGCAACCCGGGCAATTGCCCGGATCGATTTTCGTTCGTGAATACATGTTAT
```

Figure 7: Sequence analysis of the spectinomycin cassette integration site within the different GAS mutants. Black letters refer to the sequence of the corresponding streptococcal gene, red letters indicate the integrated *aad9* gene, restriction sites are underlined, and the start codon of *aad9* (Spc-cassette) is indicated in bold letters. Numbers correspond to the base pair position within the targeted gene.

### 2.3.3. Southern Blot analysis

The high sequence similarity between TCS required verification of a single insertion of the construct within the streptococcal chromosome, guaranteeing disruption of the targeted system alone. Therefore, mutants were analysed by Southern blot for multiple integrations of the spectinomycin cassette into the chromosome. Chromosomal DNA digested with *Bam*HI, *Hind*III, and *Eco*RI was hybridized with a probe specific for the spectinomycin cassette. In all cases, only a single band could be detected on the Southern blot (Fig. 8). This corresponds to a single integration of the cassette into the streptococcal chromosome.

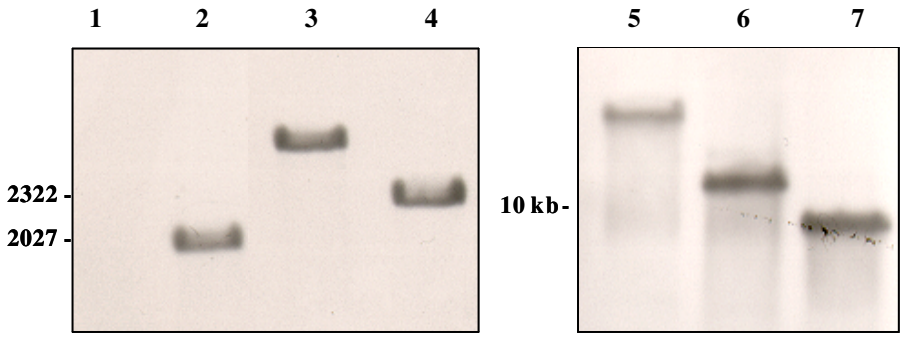



Figure 8: Southern Blot analysis of the KTL3 regulatory mutants using a probe specific for the spectinomycin cassette. 1: KTL3 wildtype; 2: ΔFasCA; 3: ΔTCS11; 4: ΔTCS13, chromosomal DNA triple-digestion with *Bam*HI, *Eco*RI, and *Hind*III; 5, 6, and 7: ΔTCS07, DNA digested with *Bam*HI (4), *Eco*RI (5), and *Hind*III (6).

## 2.4. Growth rate of the regulatory mutants

A prerequisite for a successful assessment of the role played by the TCS in bacterial virulence is the same growth behaviour of the regulatory mutants in comparison to the parental strain. Otherwise, a slower growth rate cannot be distinguished from a higher susceptibility of the mutants to host immune responses; both would result in lower bacterial counts in infection experiments when compared to the wildtype. Therefore, the growth behaviour of the mutants under standard laboratory conditions was investigated and compared to the parental strain KTL3. Representative growth curves of all mutants are displayed in Fig. 9. Under regular laboratory conditions, no obvious differences in their growth rate could be detected. Bacterial doubling times during exponential growth phase are given in Tab. 5.

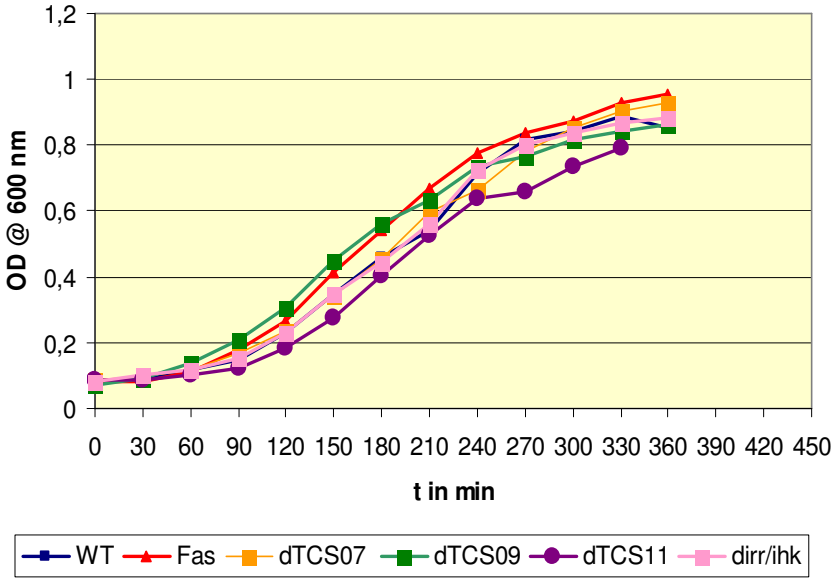



Figure 9: Representative growth curves of KTL3 wildtype and regulatory mutants.

Tab. 5: Doubling times of a representative growth curve during exponential phase of wildtype and regulatory mutants.

| **Strain** | **Doubling time [min]** |
|---|---|
| KTL3 wildtype | 74.53 |
| KTL3 ΔFasCA | 71.46 |
| KTL3 ΔTCS07 | 75.34 |
| KTL3 ΔTCS09 | 66.65 |
| KTL3 ΔTCS11 | 78.77 |
| KTL3 ΔTCS13 | 74.53 |

## 2.5. Lancefield Bactericidal Assay

The ability to survive and to grow in whole human blood in the presence of immune-relevant cells like macrophages and polymorphonuclear leukocytes (PMNs) is a prerequisite feature of *S. pyogenes* (126). During infection, this property can lead to spreading of *S. pyogenes* once it enters the bloodstream. There are several bacterial factors mediating this phagocytic resistance (see section 1.1.2.), and the ability to upregulate their expression during the encounter with host immune cells is a major virulence attribute. Consequently, when investigated *in vitro*, this ability to withstand phagocytosis can be used as indication for

streptococcal virulence. Therefore, in order to investigate the antiphagocytic ability of the regulatory mutants in comparison to the wildtype, bacteria were incubated in whole human blood as described by Lancefield (126). Impairment in the ability to survive indicated a loss of virulence and therefore a potential role of the regulatory system in regulation of antiphagocytic factors.

After three hours of incubation in fresh human blood, bacterial counts were determined and compared to the initial inoculum. Results presented in Fig. 10 show the percentage of bacteria isolated after 3 hours in comparison to 100% initial inoculum. Survival and growth of the wildtype strain within the incubation period corresponded with an increase of its number by 48 %. The TCS11 mutant was also able to grow as seen by a bacterial increase of 37.5 %. This clearly shows that their ability to resist to phagocytosis and killing is not altered. On the other hand, the bacterial number of all other mutants was reduced, the FasCA mutant by 23 %, and the TCS09 mutant by nearly 55 %. Strikingly, the Irr/Ihk- and the TCS07 mutant were almost completely eliminated, indicating a strong impact of the mutation on expression of antiphagocytic factors, leading to an impaired ability to grow and resist to phagocytosis and killing by human immune cells.

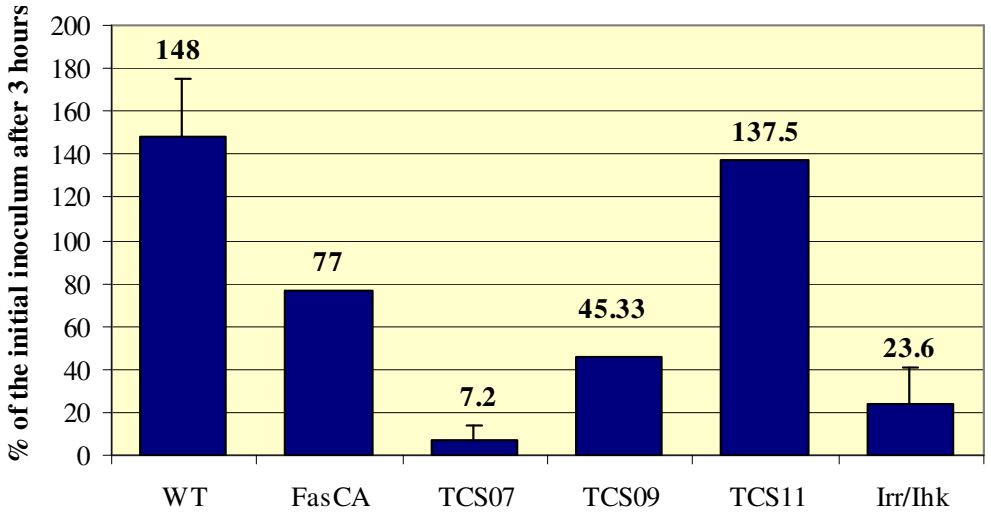



Figure 10: Bacterial counts after the Lancefield Bactericidal Assay. Results refer to 100% initial inoculum.

The Lancefield Bactericidal Assay allowed the identification of four out of five TCS which are involved in resistance against phagocytosis by host immune cells, as shown by the inability of the corresponding mutants to grow and survive when compared to the wildtype strain. The mutants deficient in TCS07 and TCS13, showing the clearest reduction within this assay were characterized in more detail regarding (i) their virulent behaviour in a murine skin infection model, and (ii) the underlying molecular mechanisms responsible for their attenuation.

### 2.6. Characterization of streptococcal TCS involved in virulence

Within the following sections, the molecular structure of TCS07 and TCS13 will be addressed and compared to similar TCS of other bacterial species. Additionally, results obtained from the characterization of the corresponding mutants regarding their virulent behaviour in different animal models, as well transcriptional, expressional, and functional analysis will be presented.

#### 2.6.1. Characterization of the TCS07 of *S. pyogenes* strain KTL3

##### 2.6.1.1. Genetic organization and structural analysis of TCS07

TCS07 is present in all so far sequenced genomes of *S. pyogenes*, i.e. M3 (15), M18 (199), M6 (12), and SSI-1 (160) (where the genes refer to *Spy*M3_0768/0769, *Spy*M18_1068/1069, M6_*Spy*828/829 (*dpiAB*), and *Sps*_0968/0969, respectively). As depicted in Tab. 3 (page 19), the histidine kinase HK07 is encoded upstream of the response regulator RR07, between these two genes, a non-coding region of 19 basepairs is located. Structurally, this system belongs to the CitAB-subfamily of two-component systems (156), which include the citrate-responsive CitAB and CitST systems from *Klebsiella pneumoniae* and *Bacillus subtilis*, as well as other C4-dicarboxylate-responsive systems, such as DcuRS and YufLM of *E. coli* and *B. subtilis*, respectively.

###### 2.6.1.1.1. Structural analysis of the response regulator RR07

The response regulator RR07 consists of 221 amino acids, encompassing all highly conserved active residues described for response regulators. In RR07, these are two aspartates at positions 8 and 9, the phospho-accepting aspartate at position 53, a lysine residue at position 103, and finally a serine located at position 81.

Prediction of the Helix-Turn-Helix (HTH) DNA-binding motif within RR07, as well as sequence alignments to known HTH motifs of other response regulators identified to 100 % a

HTH motif in RR07 starting at amino acid 175 (phenylalanine) and ending at position 199 (glutamine).

Searches for other response regulators sharing sequence homologies with RR07 revealed a large number of RRs within other bacterial species. Fig. 11 shows alignments of selected similar response regulators with RR07. All of them belong to the CitAB subfamily of two-component sensor-regulators (156) (see section 2.1.). For the response regulators CitB of *Klebsiella pneumoniae* and *E. coli,* as well as CitT of *B. subtilis,* the involvement in citrate metabolism has been demonstrated (23, 229). In *B. subtilis*, CitT controls the expression of the citrate transporter CitM (229), while in *K. pneumoniae,* it is essential for the expression of citrate fermentation genes under anoxic conditions, allowing this bacterium the growth on citrate as sole carbon source (24). In both cases, genes needed for regulation, uptake, and catabolism of citrate lie in a cluster on the bacterial genome.

The YdbG response regulator of *Bacillus subtilis* regulates transport systems for $C_4$-dicarboxylic acids such as fumarate and succinate (6). The same function has been demonstrated for DcuR in *E. coli* (77, 232). YufM of *B. subtilis* also plays a role in the utilization of a C4-dicarboxylic acid, its influence on the expression of malate transporters MaeN and YflS could be demonstrated; furthermore, it activates the transcription of the malic enzyme gene *ywkA* in response to malate (52, 210).


Figure 11: Sequence alignment of RR07 of *S. pyogenes* strain SF370 with similar response regulators of other bacterial species. Spy = *S. pyogenes*; Eco = *E. coli*; Kpn = *K. pneumoniae*; Sco = *S. coelicolor*; Bsu = *B. subtilis*. Conserved residues are indicated in red, HTH-DNA-binding motifs are shaded in grey.

### 2.6.1.1.2. Structural analysis of the histidine kinase HK07

The sensor kinase HK07 is composed of 513 amino acids comprising three functional domains. Due to characteristic patterns found within its homology boxes, it can be grouped into the HK5 subfamily of histidine kinases (82). Structurally, HK07 shows all the characteristics for sensors of the CitAB-subfamily of TCS. It has two membrane-spanning helices in the N-terminal region, the first one ranging from amino acid 11 to 27, the second one from amino acid 173 to 192, resulting in an extracellularly located sensing domain of 146 amino acids. Comparisons of this domain with protein databases did not identify homologies to known proteins; therefore no predictions about putative signals could be made. Additionally, protein family scans revealed the presence of a PAS domain (PFAM: PF00989), encompassing amino acid 211 through 243 in HK07. These domains, which are often present in histidine kinases, mediate the sensing of intracellular signals, such as oxygen levels,

temperature, or acidic conditions (212). In the CitA-like histidine kinases of gram-negative bacteria, they have been shown to act as a periplasmatically located sensor for tri- and/or dicarboxylates (112, 166, 181). Therefore, besides the extracellularly located domain, the PAS domain of HK07 might play an additional role in signal perception. The transmitter domain of HK07, facilitating the transfer of the phosphoryl group to the cognate response regulator, is located from amino acid 327 to 513.

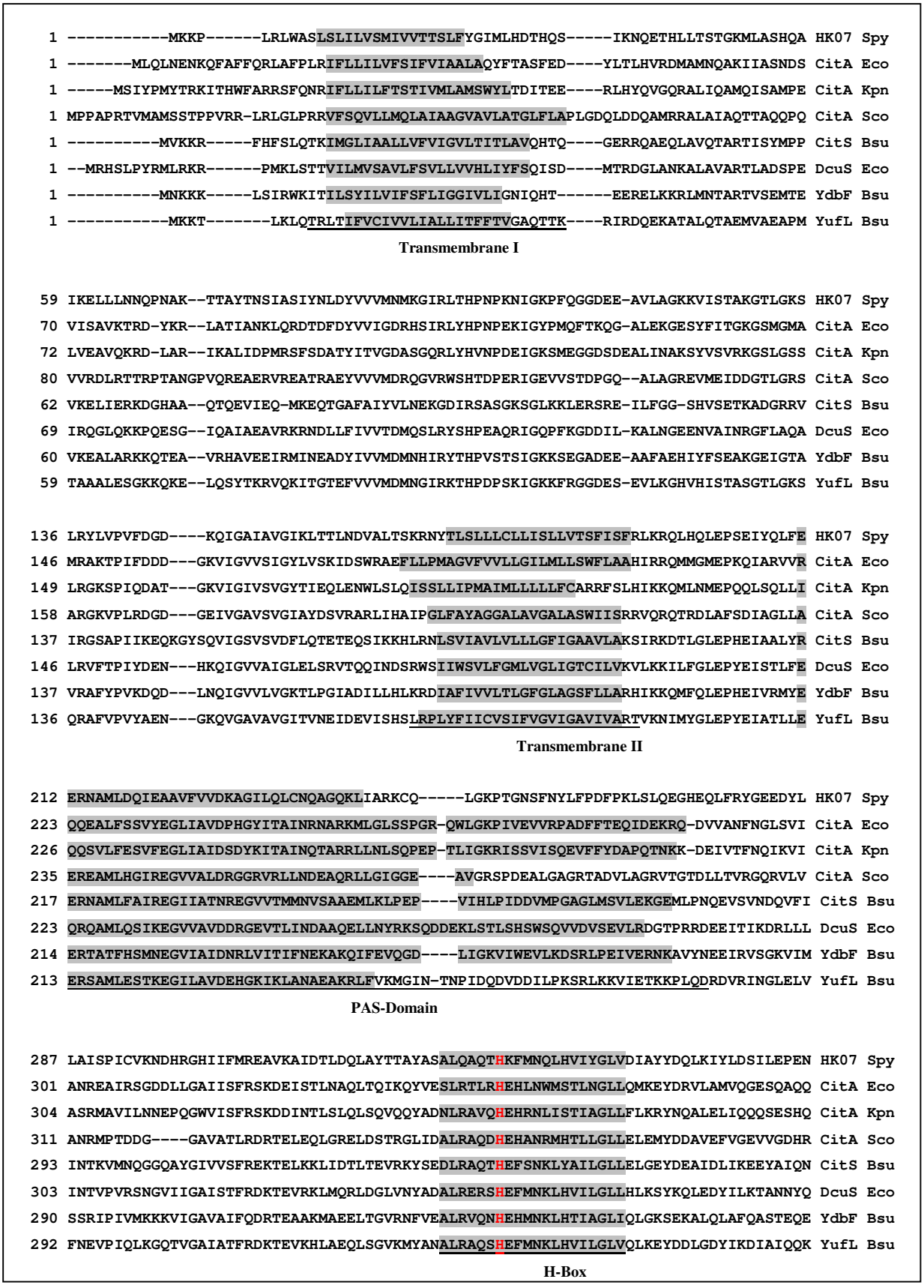
Scans for the typical histidine kinase signature sequences clearly identified four out of the five homology boxes. The H-Box of HK07 ranges from amino acid 327 to 346, harbouring the crucial phospho-accepting histidine at position 333. The conserved asparagine lies within the N-Box (amino acid 423-434) and is located at position 429 of HK07. The α-helical X-region ranges from amino acid 372 to 395, however the lysine within this region is missing in HK07. This residue can be found in members of the HK5 subfamily, in other HK-subfamilies it is often absent and therefore its function remains unclear (82). The D-Box with its conserved aspartate (amino acid 462) ranges from position 457 - 462, whereas the phenylalanine-containing F-Box spans the regions from amino acid 469 through 471. The G-Box of HK07 is partly missing; only two residues are present, represented by two glycines at position 505 and 506. Therefore, HK07 could exhibit an impaired ability or an alternate mechanism for ATP binding, since the G-Box in involved in this process. Sequence comparisons of HK07 revealed the same incomplete G-Box within the other sequenced *S. pyogenes* strains, therefore sequencing errors in HK07 of strain SF370 can be excluded. All homology boxes, as well as the X-region are indicated in Fig. 12, an additional overview is given in Tab. 6.

Table 6: Positions of homology boxes found in HK07 with and conserved residues.

| **Homology box / region** | **Amino acid positions** | **Conserved residue and position** |
|---|---|---|
| H-Box | 327 – 346 | Histidine 333 |
| X-Region | 372 – 395 | Lysine missing |
| N-Box | 423 – 434 | Asparagine 429 |
| D-Box | 457 – 462 | Aspartate 462 |
| F-Box | 469 – 471 | Phenylalanine 471 |
| G-Box | Partly missing | Glycine 505 and 506 |

Homology searches of HK07 with bacterial genome databases identified the cognate histidine kinases to the in 2.6.1.1.1. described response regulators of the CitAB-subfamily, as well as a similar HK of unknown function in the closely related species *Streptococcus agalactiae.* Sequence alignments of HK07 with these histidine kinases are displayed in Fig. 12, HK-

specific signature sequences, as well as PAS- and transmembrane domains are indicated. The partly missing G-Box of HK07 is however present in the homologues.



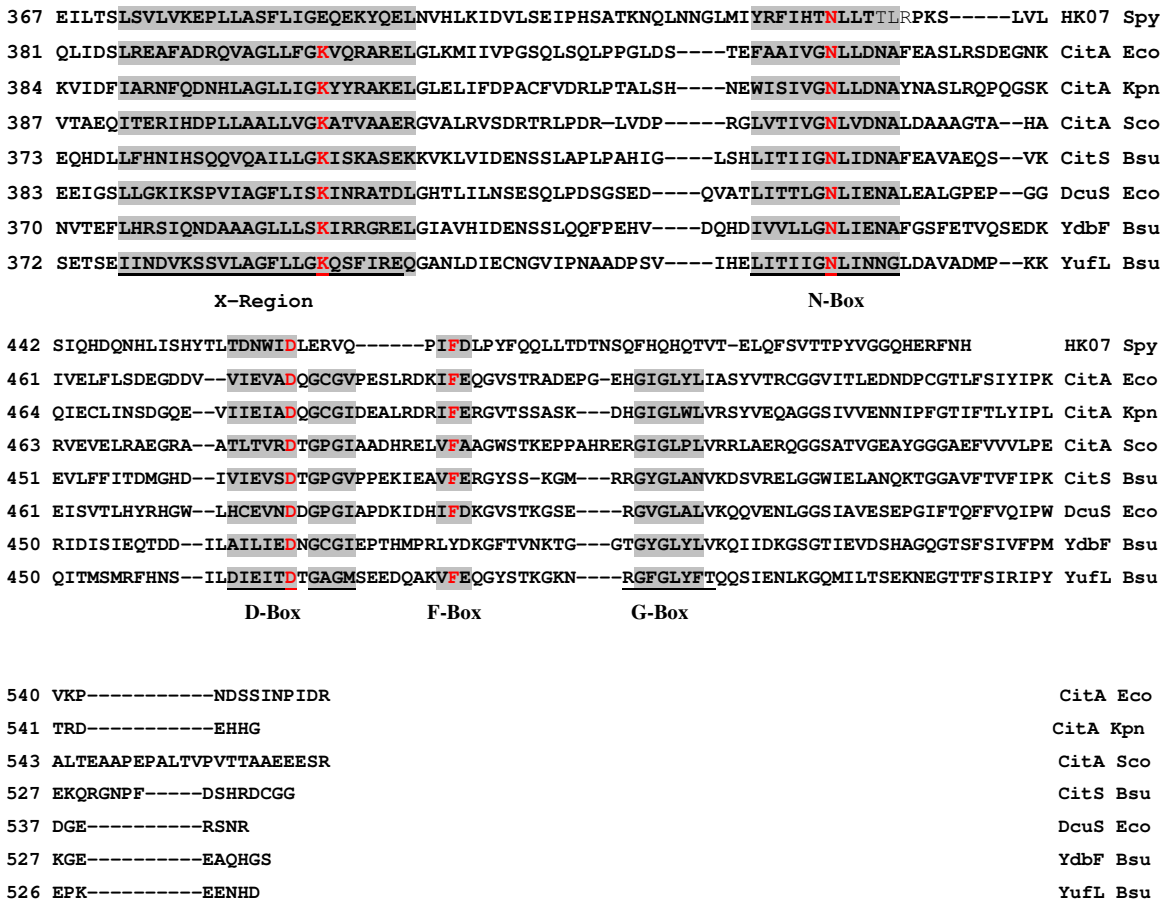



Figure 12: Sequence alignment of HK07 of *S. pyogenes* strain SF370 with similar histidine kinases from other bacterial species. Spy = *S. pyogenes*; Eco = *E. coli*; Kpn = *K. pneumoniae*; Sco = *S. coelicolor*; Bsu = *B. subtilis*. Conserved residues are indicated in red, signature sequences, transmembrane helices, and PAS domains are shaded.

### 2.6.1.2. Chromosomal neighbourhood of the TCS07

Compared to adjacent lying genes, TCS07 is transcribed in the opposite direction and therefore insertion of the spectinomycin cassette will not have any influences on the transcription of downstream-located genes. Fig. 13 gives a more detailed overview of the chromosomal neighbourhood of TCS07, which is identical in all so far sequenced *S. pyogenes* strains, but different in closely related species such as *S. agalactiae*. Downstream of *rr07* - and transcribed in opposite direction - a putative ABC-transporter of the spermidine-/putrescine family (75) is located. In *E. coli*, this type of system mediates the transport of polyamines (75, 112). A putative malate permease and malic enzyme (EC 1.1.1.39) are encoded upstream of the histidine kinase *hk*07, of which homologues in *S. bovis* have been

investigated in more detail (114, 132). There, the malic enzyme catalyzes the oxidative decarboxylation of L-malate, as well as the reductive carboxylation of pyruvate. L-malate permease, on the other hand, is cotranscribed with the malic enzyme, and functions as L-malate specific permease that is highly active at low pH (114, 115).

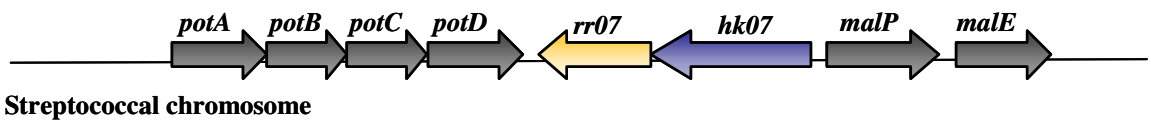



Figure 13: Chromosomal neighbourhood of *tcs07*. *PotA-D* refer to structural genes of the ABC transporter, *malP* and *malE* encode the malic enzyme and -transporter.

### 2.6.1.3. Growth characteristics of the TCS07 mutant

In 3.1., it was demonstrated that the TCS07 mutant does not show any difference in its growth rate when compared to the parental strain. Nevertheless, a different growth phenotype was observed, i.e. altered sedimentation behaviour. Additionally, due to the described regulatory functions of similar TCS in other species, the effects of different C-sources and other variations of growth parameters were analysed.

#### 2.6.1.3.1. Sedimentation behaviour

During growth in standard broth, differential sedimentation behaviour of the ΔTCS07 strain was observed in comparison to the wildtype. Once reaching early- to mid-logarithmic growth phase, the parental strain formed aggregates and started to sediment, the mutant strain however did not (Fig. 14). Frick *et al.* (73) investigated sedimentation behaviours of different GAS serotypes and identified a conserved region of 19 amino acids present in the streptococcal M- and M-like protein H. They concluded that homophilic protein-protein interactions between neighbouring proteins of *S. pyogenes* containing this region caused sedimentation in liquid medium. Additionally, aggregation of *S. pyogenes* was accompanied with an increased ability to survive in whole human blood. (73). The lack of sedimentation observed in TCS07 mutant cultures may therefore be due to a lower expression of M-protein or other AHP-containing surface proteins. The analysis of M protein expression on the surface of wildtype and TCS07 mutant will be presented in 2.6.1.5.3.

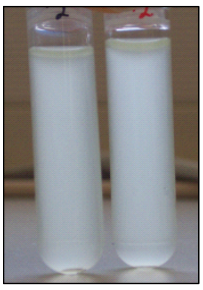
WT ΔTCS07

Figure 14: Sedimentation behaviour of KTL3 and its isogenic mutant ΔTCS07.

### 2.6.1.3.2. Exchange of the C-Source

Due to the close relationship of the TCS07 system to other regulatory systems involved in the utilization of citrate, malate, succinate, and fumarate, the ability of wildtype and ΔTCS07 strain to grow on these compounds (as well as pyruvate) was addressed. However, neither wildtype, nor mutant were able to grow in medium containing these compounds as sole C-source, except when glucose was supplemented in addition. Growth on lactose as sole carbon source occurred after an extensive lag phase of approximately 3.5 hours, but again, no detectable differences could be observed between parental and mutant strain.

Interestingly, no phenotypic difference in the sedimentation behaviour of wildtype and TCS07 strain cultures could be observed when grown in medium containing glucose and malate. Neither Wildtype, nor mutant cultures showed aggregative behaviour and sedimentation (Fig. 15). This stands in contrast to observations made in regular growth medium (Fig. 14), or in medium supplemented with other C-sources, where the wildtype invariably sedimented, but the mutant did not.



WT ΔTCS07

Figure 15: Sedimentation behaviour of KTL3 wildtype and isogenic TCS07 mutant in CDM-glucose, supplemented with malate.

#### 2.6.1.3.3. Dependency of pH on growth ability

After phagocytosis by cells of the host's immune system, bacteria are enclosed in phagosomes, which is followed by a fast drop in pH (54). The inability to survive within the Lancefield Bactericidal Assay could be directly linked to an incapability to respond to such changes in pH. Therefore, in order to investigate the possible role of HK07 in sensing and responding to an increased level of protons or ions, the ability of wildtype and TCS07 mutant to grow at different pH levels was investigated. Results summarized in Tab. 7 demonstrate that bacterial growth required a minimum pH of 5.5, at all other investigated pH levels, no difference between wildtype and TCS07 mutant was observed. These results demonstrate the equal ability of both strains to grow at low or high pH.

Table 7: Influence of pH on bacterial growth.

| **pH** | **WT** | **ΔTCS07** |
|---|---|---|
| pH 5.0 | - | - |
| pH 5.5 | + | + |
| pH 6.0 | ++ | ++ |
| pH 6.5 | +++ | +++ |
| pH 7.0 | +++ | +++ |
| pH 7.5 | +++ | +++ |
| pH 8.0 | ++ | ++ |
| pH 8.5 | ++ | ++ |

+++ represents very good growth; ++ good growth; + poor growth; - no growth.

Taken together, growth analysis of wildtype and ΔTCS07 strain in the present of diverse C-sources, as well as varying pH levels did not show any significant differences, even though structural similarities to other TCS involved in the utilization of C4-compounds exist. The only striking observation was a lack of sedimentation in the wildtype strain when malate was supplemented, a phenotype that so far has been a characteristic for the ΔTCS07 strain.

### 2.6.1.4. Contribution of the TCS07 to bacterial virulence in a mouse model of infection

As already shown in 2.3.2., the mutant exhibited an impaired ability to survive in whole human blood. However, *in vitro* experiments do not necessarily reflect the situation occurring during *in vivo* infections, where the interactions of bacteria with their host are more complex. Therefore, the virulence of the TCS07 mutant was investigated in a mouse model of subcutaneous infection and compared to that of the wildtype strain.

#### 2.6.1.4.1. Survival time of infected animals

One method to assess bacterial virulence is to monitor the animal's survival after infection. Therefore, mice were subcutaneously infected either with KTL3, or with the ΔTCS07 isogenic mutant strain and the survival time of individual animals was determined over time. A Kaplan-Meier Plot of one representative experiment is shown in Fig. 16. By day three, about 50 – 60 % of wildtype-infected animals succumbed to infection, by day five, 100% mortality was observed within this group. In contrast, mice infected with an equal amount of ΔTCS07-bacteria survived over the whole investigated time-period, clearly showing the attenuation of the regulatory mutant in comparison to the wildtype strain.

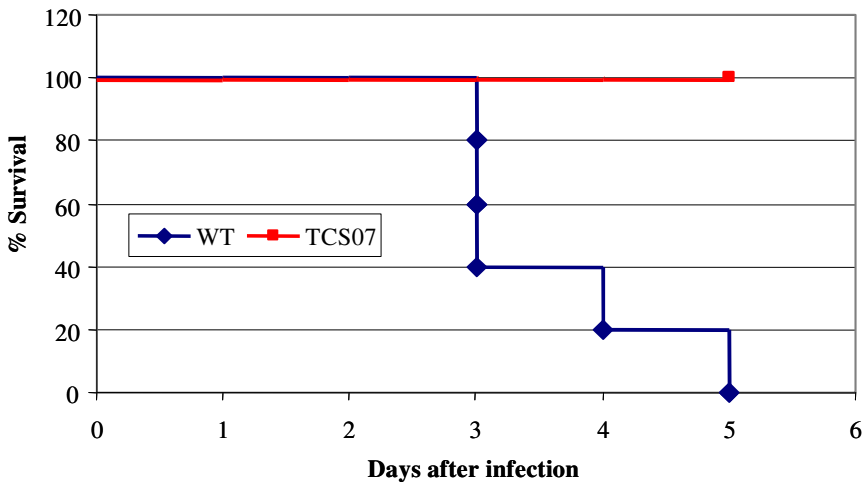



Figure 16: Survival of mice infected with either KTL3 wildtype or ΔTCS07.

#### 2.6.1.4.2. Determination of the lesion sizes

In order to estimate the virulent behaviour of wildtype and mutant at the local site of infection, the sizes of the skin lesions were measured 48 hours postinfection. Animals infected with the wildtype developed lesions of about 3.1 $cm^2$, whereas in mice inoculated with the ΔTCS07 strain only very small lesions of about 0.2 $cm^2$ were observed. Therefore, the mutant has a decreased ability to induce lesions in infected animals.

#### 2.6.1.4.3. Dissemination of bacteria from local infection foci to systemic organs and bloodstream.

The possibility that the observed attenuation of the mutant could be due to a reduced bacterial ability to spread from the local site of infection through the bloodstream and to establish a systemic infection was further investigated. For this purpose, bacterial loads of wildtype and mutant were determined in blood, as well as in systemic organs of infected mice. Mice were infected with either wildtype or ΔTCS07, and 24, 48, and 72 hours after subcutaneous infection, liver, spleen, and blood samples were taken, serially diluted in PBS and plated in order to determine the exact bacterial number.

During the infection period, bacterial loads increased steadily in systemic organs of wildtype-infected mice (Fig. 17). After 24 hours, no bacteria were detectable in the bloodstream; most bacteria were located in liver and spleen ($log_{10}$ 3.29 and $log_{10}$ 2.68), respectively. After 48 hours, bacterial counts within systemic organs increased in the wildtype, reaching $log_{10}$ 6 in liver and spleen, and $log_{10}$ 4.2 in blood (Fig.17). By day 3, all wildtype-infected animals had died, indicating an inability of these animals to control the infection.

In contrast, mice infected with the TCS07 mutant had fewer bacteria in blood and systemic organs at all time-points investigated. After 48 hours, an increase of bacteria could be observed, but never exceeded $log_{10}$ 3. By 72 hours, all bacteria were eliminated from blood and systemic organs of infected animals. These results show the ability of mice infected with the TCS07 mutant to clear the infection from bloodstream and systemic organs at 72 hours postinfection.

Over all, the outcome of the animal infection parallel results obtained from the Lancefield Bactericidal Assay (2.5). Animals infected with ΔTCS07 showed a reduced virulence in a mouse model of skin infection. This could be observed by survival of animals during the monitoring-period, as well as by the development of smaller skin lesions and a rapid clearing

of the mutant from blood and systemic organs.

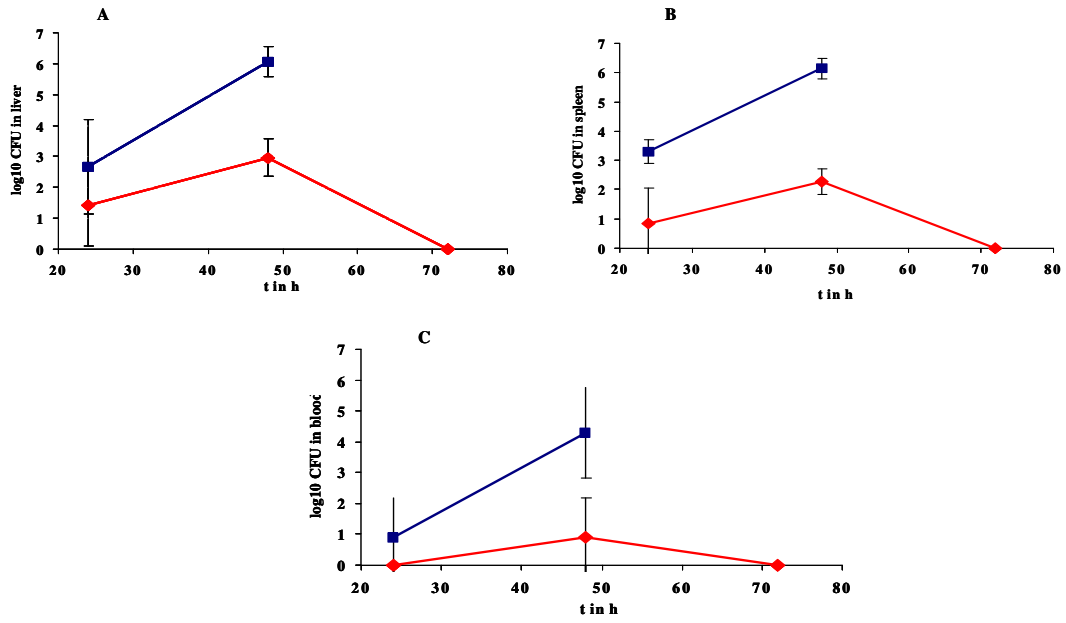



Figure 17: Distribution of KTL3 wildtype and ΔTCS07 bacteria to mouse organs and blood at 24, 48, and 72 hours postinfection. KTL3 is displayed in blue, ΔTCS07 in red. (A) Bacteria isolated from spleen. (B) Bacteria isolated from liver. (C) Bacteria isolated from blood.

### 2.6.1.5. Identification of differentially expressed proteins and virulence factors

Reduced virulence in the Lancefield Bactericidal Assay (2.5.), as well as in *in vivo* infections can have a variety of reasons. Streptococcal knock-out mutants missing M-protein have been shown to exhibit loss in virulence (168). Such high attenuation in animal models as observed for the ΔTCS07 strain has only been reported in the absence of major virulence factors, such as the hyaluronic acid capsule, M protein, and to some degree SpeB (8, 223). Thus, to understand and identify the molecular basis of the reduced virulence in the TCS07 mutant strain, the expression levels of these important virulence factors was investigated in ΔTCS07 and compared to the wildtype strain.

#### 2.6.1.5.1. Hyaluronic acid capsule

The amount of cell-associated capsule can be determined by an unspecific staining method in combination with measurement of photometric absorbance. Capsule is usually upregulated during early exponential growth phase; its expression reaches a peak in logarithmic growth, and decreases towards stationary phase (45).

Blood passage of *S. pyogenes* can result in increased capsule expression, visible by highly mucoid colonies (178, 180). The same observation could be made for KTL3 after subjection to blood-passage or when isolated from the liver of infected animals. ΔTCS07-colonies rising from organs of infected animals, as well as from mouse- or human blood were unencapsulated, observable by a missing mucoid phenotype (Fig.18 D). For quantification, the amount of cell-associated capsule was determined from those colonies. The parental strain KTL3 showed a capsule expression that was 6.8 fold higher than in ΔTCS07 (89 fg/cfu to 13 fg/cfu, respectively), in which only minor amounts of capsule could be detected (Tab.8). This difference was not visible under standard culture conditions (Fig. 18 A and 18 C), but only when bacteria were isolated from blood or systemic organs.

Mouse-passages often result in selection of spontaneous mutants in the capsule synthesis regulating gene locus, CsrRS, accompanied by isolation of *S. pyogenes* strains with a highly mucoid phenotype (59). For excluding the possibility that the mucoid phenotype of the wildtype strain was due to mutation, encapsulated wildtype GAS were subjected to several broth-passages. Loss of the capsule was achieved after 2-5 passages, confirming this phenotype to be revertible and therefore not due to a spontaneous mutation. These results clearly show than in the wildtype strain, an upregulation of hyaluronic acid capsule occurs within blood and organs. This upregulation is absent in the TCS07 mutant, i.e. the mutant is no longer able to respond to an environmental stimulus present in blood or organs.

Consequently, TCS07 is involved in either positively activating capsule transcription directly, or in regulating genes essential for efficient expression of this virulence factor under stress conditions.

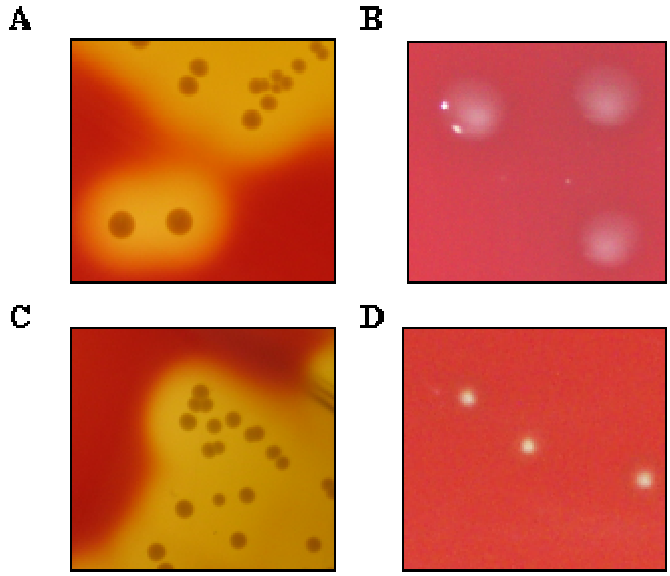



Figure 18: KTL3 (A) and ΔTCS07 (C) colonies on blood agar plates grown under standard conditions. Mucoid KTL3 (B) and non-mucoid ΔTCS07 isolated from mouse liver and subsequently grown on blood agar plates..

Tab. 8: Amount of hyaluronic acid capsule of bacteria isolated from mouse blood and subsequently grown on blood agar plates.

| | **Hyaluronic acid capsule [fg/CFU]** |
|---|---|
| KTL3 WT | 89 ± 0.71 |
| KTL3 ΔTCS07 | 13 ± 2.83 |

### 2.6.1.5.2. Streptococcal inhibitor of complement (sic)

For detection of differences in the pattern of secreted proteins from wildtype and TCS07 mutant, precipitated supernatants of stationary grown cultures were separated on a 1-dimensional SDS gel. As seen in Figure 19, three bands are visible in the wildtype strain KTL3, of which two are absent from the supernatant of the TCS07 mutant. The first missing protein has a molecular mass of ~ 100 kDa, the second one is about 45 kDa in size. Isolation of the respective bands from the SDS gel and subsequent Maldi-TOF analysis could not identify the 100 kDa proteins, but the ~ 52 kDa-sized proteins present in both strains were identified as a putative secreted protein, corresponding to SF370 gene number *Spy*0019. The

45 kDa proteins lacking in the ΔTCS07 strain, on the other hand, were found to be the well-known streptococcal virulence factor SIC. This protein is a GAS secreted protein that interferes with complement-mediated lysis and is predominantly present in *S. pyogenes* of serotypes M1 (2; see section 1.1.2.3).

These results clearly show the absence of the virulence factor SIC in the TCS07 mutant.

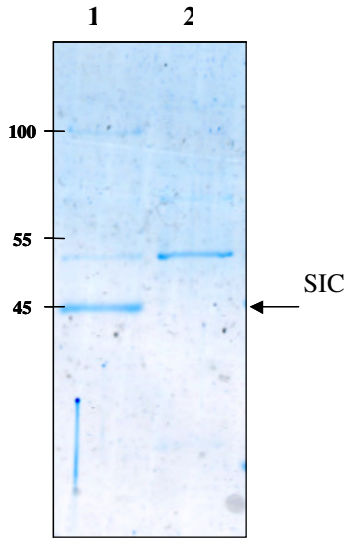



Figure 19: Analysis of precipitated secreted proteins in KTL3 and ΔTCS07. SDS gel with supernatants from wildtype (lane 1) and TCS07 mutant (lane 2). Maldi –TOF identified protein SIC is indicated by an arrow. Sizes are indicated on the left in kDa.

### 2.6.1.5.3. Detection of M1-Protein

Besides capsule, M-protein is one of the major and best described streptococcal virulence factors (68; see section 1.1.2.2.). Since the regulatory mutant exhibited a significant loss in phagocytic resistance and in murine virulence, a putative impact of the missing TCS on the amount of M1-protein was investigated. For preventing M protein degradation by SpeB, bacteria were grown in standard broth in the presence of a cysteine protease inhibitor. Subsequently, M1 extraction from the surface of KTL3 and ΔTCS07 and Western analysis with anti-M1 serum could detect slightly degraded M protein in the wildtype, but none in the mutant (Fig. 20). Hence, besides the incapability to upregulate capsule in blood and organs, the TCS07 mutant's attenuation is additionally linked to the lack of the M1 protein. Likewise, these results go conform to the slower sedimentation exhibited by stationary-grown ΔTCS07 (2.6.1.3.1.), since low expression rates of M-protein have been associated with this phenotype (21, 73).

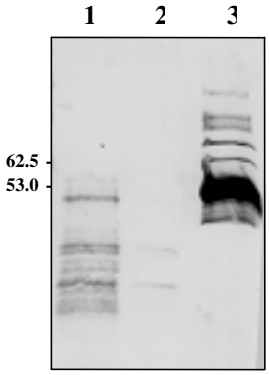



Figure 20: Western blot analysis of the expression of M1-protein in wildtype and TCS07 mutant, detected by incubation with polyclonal anti-M1 serum. 1. HCl-extraction of KTL3 2. HCl-extraction of ΔTCS07. 3. Recombinant M1 protein. Sizes are indicated on the left in kDa.

To further prove this hypothesis, field emission electron microscopy (FESEM) for detection of surface-located M protein was performed. Purified anti-M1 antibody, which was subsequently gold-labeled, was incubated with wildtype and ΔTCS07. FESEM photographs displayed in Fig. 21 show images of wildtype (A) and ΔTCS07 (B). Due to the gold-label, surface-localized M proteins appear as white dots. As already seen by Western analysis, the wildtype expresses adequate amounts of M protein. In contrast, ΔTCS07 produces only little or none of this virulence factor.

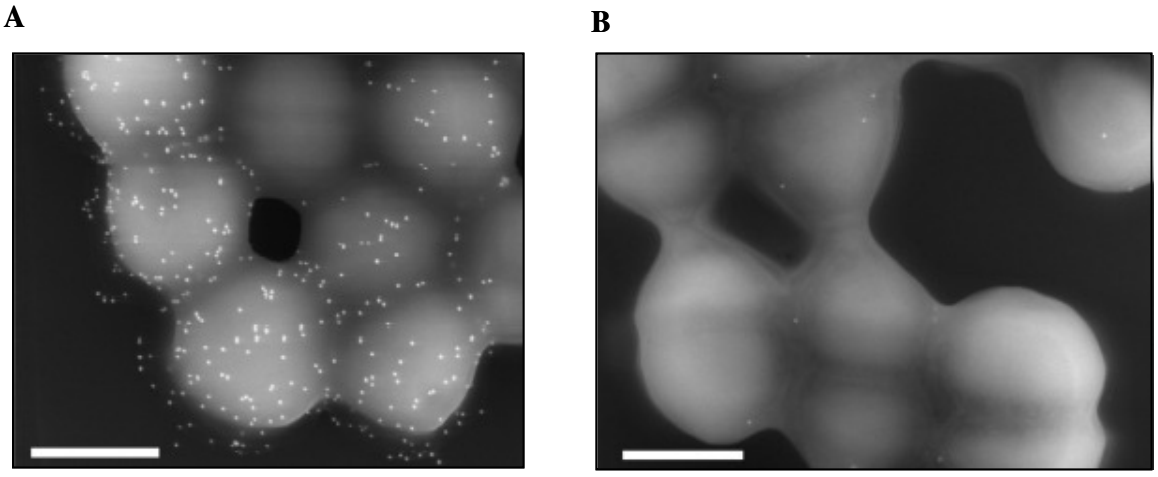



Figure 21: FESEM picture of surface-localized M1 protein on KTL3 (A) and ΔTCS07 (B). Bar corresponds to 0.5 µM.

The ability to bind to human fibrinogen is a streptococcal attribute that has not only been ascribed to the M1-protein, but to many other streptococcal proteins such as FnBP54, serum

opacity factor (SOF), and protein F (see section 1.1.1.2). For strain KTL3 it was reported that fibrinogen binding is solely due to the M1-protein (179). Therefore, it was investigated whether the lack of M1-protein in ΔTCS07 impairs the mutant's ability to bind to radiolabelled human fibrinogen. As seen in Fig. 22, a clear reduction of the capability to bind to human fibrinogen was observed for the TCS07 mutant, which was only able to bind 23.6 % of the added fibrinogen. In the parallel experiment, the parental strain KTL3 bound 94.1 % of deployed labelled protein (Fig. 22). Therefore, the lack of the M1-protein in the regulatory mutant clearly reduces its ability to bind to human fibrinogen.

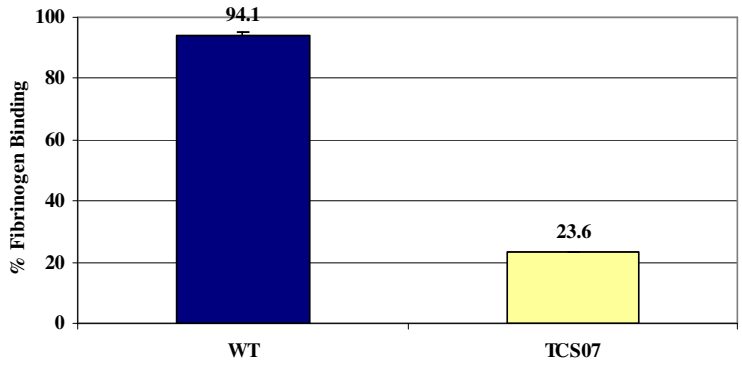



Figure 22: Binding of radiolabelled human fibrinogen to wildtype and ΔTCS07 shown as percentage of the total amount of deployed protein.

### 2.6.1.6. Transcription analysis of streptococcal genes

#### 2.6.1.6.1. Virulence factors *emm, sic,* and *hasA*

For further elucidating the nature of the difference seen for the M1-protein and SIC in the TCS07 mutant, transcriptional analysis of these virulence factors was performed by Northern blot. For *emm1*, as well as for *sic* no transcript was detected in the TCS07 mutant (Fig. 23 A and B, lane 2). In contrast, strong signals were detected in KTL3 (Fig. 23 A and B, lane 1). Reprobing of both blots with a specific probe for the house-keeping gene *rpsL* confirmed loading of equal amounts of RNA (Fig. 23C). Consequently, the lacking expression of M protein and SIC in ΔTCS07 were due to absent transcripts. Both virulence factors are under positive regulatory control of Mga (149), thus the observed effects could be caused by an influence of TCS07 on the transcription of *mga*. Northern analysis of this single regulator however did not yield any signals in both, wildtype and mutant. Likewise, at no time-point

was a signal detectable for *hasA*, representing the capsule gene cluster, which was probably due to amounts of transcripts below detection limits. Therefore an indifferent transcription of hyaluronic acid capsule, as well as *mga* could not be shown.

The obtained results demonstrate that the observed differences in protein level seen for M protein and SIC (see section 2.6.1.5.2. and 2.6.1.5.3.) are due to a lack of transcription in the TCS07 mutant, and not to post-translational modifications. Consequently, TCS07 is directly or indirectly involved in the transcription of these streptococcal virulence factors. An indirect influence of the transcriptional regulator Mga on the observed results, however, cannot be excluded.

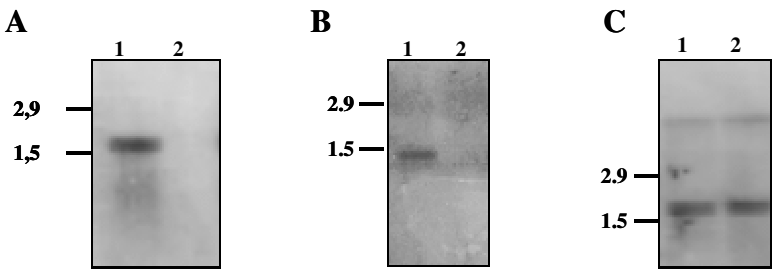



Figure 23: Transcriptional analysis of virulence factor genes *emm1* (A) and *sic* (B) in KTL3 (lane 1) and ΔTCS07 (lane 2) by Northern blot. Loading of equal amounts of RNA was confirmed by reprobing the Northern blot with an rpsL-specific probe (C). Transcript sizes are indicated on the left.

Additional transcription analysis was performed to assess amount of *emm1*-transcript as possible reason for the lost sedimentation of the wildtype strain that was only observed in malate-containing medium (2.6.1.3.2.). In the wildtype strain, a repression of *emm1* transcription mediated by TCS07 in the presence of malate and consequently lower levels of M protein – as factor inducing sedimentation by homophilic protein interactions (2.6.1.3.1) - could explain this observed lack of sedimentation. However, Northern analysis of KTL3 grown in the presence of malate showed a detectable *emm1*-transcript (Fig. 24), and therefore indicating the presence of M1-protein under malate-containing conditions.

### 2.6.1.6.2. Neighbouring genes of TCS07

The wildtype exhibited the same sedimentation phenotype as the TCS07 mutant only in CDM supplemented with malate. Consequently, it seemed tempting that the regulatory circuit

leading to this phenotype might be similar in wildtype and mutant under these conditions. The involvement of malic- enzyme and permease in malate uptake and catabolism has been shown in *S. bovis* (114, 115), and the corresponding genes *malE* and *malP* are located adjacent to TCS07 on the GAS chromosome (Fig. 13). Thus, the regulation of these malate-associated genes was investigated by Northern analysis with RNA isolated from bacteria grown in the presence of this C4-compound. However, no transcript of the calculated size of 1.3 kb was detectable. The same results were obtained for transcriptional analysis of the TCS-neighbouring ABC putrescine-/spermidine transport system (*potA-D*). In summary, the growth in malate-containing medium did neither induce detectable levels of malic enzyme and L-malate permease, nor of the downstream located ABC-transporter.

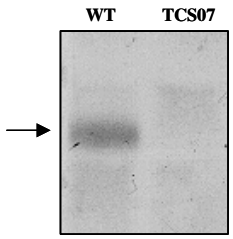



Figure 24: Northern blot analysis of emm1 in KTL3 and TCS07 mutant grown in CDM supplemented with malate (4.1.3.2.). An arrow indicates the *emm*1 transcript.

### 2.6.2. Characterization of the TCS13 (Irr/Ihk) of *S. pyogenes* strain KTL3

#### 2.6.2.1. Genetic organization and structural analysis of TCS13

TCS13 has been named Irr/Ihk (isp-adjacent histidine kinase/response regulator) by Federle *et al*. (63), who partially characterized a TCS13 deletion strain in an M6 background with respect to transcription of the virulence factors M protein, C5a peptidase, SLO, SLS, streptokinase and capsule synthetase, as well as the regulator Mga. However, they neither found any differences between wildtype and mutant strain, nor did not test the mutant for attenuated virulence in animal models.

In the annotated streptococcal genome SF370, TCS13 has been assigned as *Spy*2026/2027. It is present in all annotated Group A streptococcal genomes (corresponding genes: *Spy*M3_1732/1733 (15), M6_*Spy* 1724/1725 (12), *Spy*M18 2083/2084 (199), and *SPs*_1729/1730, respectively (160), and shares 99 - 100 % identity on the protein level. Structurally, TCS13 belongs to the OmpR family of two-component systems and has the response regulator located upstream of the histidine kinase, as it is typical for this family of TCS. A Shine-Dalgarno sequence is located prior to the start codon of RR13, and displayed Figure 25, –10 and –35 regions as probable binding sites for the RNA-polymerase are also indicated. The region in between the stop-codon of the response regulator and the start-codon of the histidine kinase encompasses merely 3 bp. In the region downstream of HK13, scans revealed no putative inverted repeats as transcriptional terminators. However, by extending scans to the downstream located gene *isp*, an incomplete palindrome could be identified (Fig. 25). The resulting stem-loop would be composed of 11 basepairs, with 2 imperfect matches of guanine with uracil. However, due to this weak terminator structure, it cannot be excluded that the transcription of the succeeding gene *isp* is influenced in the TCS13 mutant.


Figure 25: Abbreviated sequence of the TCS13: "start RR13 " corresponds to the beginning of the response regulator, "stop HK13 " to the end of the histidine kinase, and "start isp" refers to the beginning of the following gene. Shine-Dalgarno (SD), -10, and –35 region are underlined, inverted repeats (IR) as putative transcriptional terminator are displayed in red, mismatches within the IR in bold letters.

#### 2.6.2.1.1. Structural analysis of the response regulator RR13

Sequence analysis of the response regulator RR13, which consists 218 amino acids, revealed all five characteristic conserved residues (see section 1.2.) to be present in RR13. The conserved asparagines in the N-terminal receiver domain are located at amino acid positions 9 and 10 of RR13, the phospho-accepting aspartate at position 52, whereas within the C-terminal regulatory domain, the conserved threonine is located at position 79, and the lysine at position 101. The sequence of RR13 with all its five conserved residues is represented in Fig. 26.

Homology searches and comparisons of the C-terminal region of RR13 revealed the affiliation of this response regulator to the OmpR family. OmpR itself has been characterized in great detail and shown to control the porin expression system in *E. coli* (204). The crystal structure of the C-terminal domain has been resolved resolved and revealed a 'winged helix-turn helix motif' as DNA binding motif (144). It has been demonstrated that response regulators of this family are crucial for a broad range of regulatory functions, like antibiotic resistance (VanR of *Enterococcus faecalis* (61), phosphate regulation (PhoP of *B. subtilis*; 193), as well as regulation of secreted virulence factors (ArlR of *S. aureus*; 72)

Searches for response regulators sharing sequence homologies to RR13 identified RRs belonging to the OmpR family, as well. These include a so far non-characterized response regulator in *S. suis*, as well as a number of regulators of the *Bacillus* genera, with yet

unknown functions. VanR of *Enterococcus faecalis* is with 49 % identity the most similar response regulator with known functions. It has been shown to bind to a promoter region within the vancomycin resistance gene cluster of *E. faecalis,* leading to a change in the expression of genes required for this bacterium to grow in the presence of the corresponding antibiotic (61). A sequence alignment of RR13 with these response regulators is given in Fig. 26.

```
  1 -MFKILVVEDDDTISQVICEFLKANNYDPDCVFDGQAALDKWQTTSYDLIILDIMLPSLSGLEVLKTIRKTSDVPIIMLT Spy RR13
  1 -MTHILVIEDNPDIQELIREFLMAQNFTVDVVGAGTEGILLFQKNSYDLVLLDVMLPDIDGYSICKIMRGQSDVPIIMLT Bce vanR
  1 MSIRILLVEDDDHICNTVRGFLAEAGYQVDACTDGNEAYTKFYENTYQLVILDIMLPGMNGHELLREFRAKNDTPILMMT Efa vanR
  1 -MEKILIAEDDSAILGVITAFLTEAGYQVMTAKNGIEAYHLFQKETFDLIIMDIMMPSMDGYTLTELIRSTSTTPILMMT Lmo phoP
  1 -MSRILVVEDDIVISQVVCEFLKEHGYQVESVFDGKVALERFQEEQFDLIVLDIMIPSMTGLEVLKEIRKTSQIPILMLT Ssu

 80 ALDDEYTQLVSFNHLISDYVTKPFSPLILIKRIENVLRV---STPDEK-----RQIGDLLVDETEHSVYWQGTLVKLTKK Spy RR13
 80 GLHNEESEIKGFELGIDDYITKPFHYTVFIKRVEAVLRR---AATKEAEAATILQFHELMLNSTAYAAYVHGNQIELTTK Bce vanR
 81 ALSDDENQIRAFDAEADDYVTKPFKMQILLKRVEALLRR---SGALAK----EIRVGRLTLLPEDFTVLCDGTELPLTRK Efa vanR
 80 ALSEEDDELKGFDLGADDYIQKPFSYLVLLKRVQVLLRRVNQSETKKQ----ILRCGNISVDTETFEVISAGETIELTKK Lmo phoP
 80 AMGDEYTQLISFNQIISDYVVKPFSPTILVKRIENILRG---KGETDS-----IEIGTILIQPTSGAVYMEEEEVQLTKK Ssu

152 EYDIIDYLAKRHQKIVTRDQLMDDIWGYSEL-DTRVLDNHIKNLRKKMTGIPLKTITGMGYLLGERE               Spy RR13
157 EFEIIHTLLQNRGKVLSRSDLLNKIWGYEHYGDVRVIDTHIKNLRKKLGIPYIKTVKGIGYKIES               Bce vanR
154 EFEILLLLVQNKGRTLTHEIILSRIWGYDFEGDGSTVHTHIKNLRAKLPENIIKTIRGVGYRLEESL             Efa vanR
156 EFDILVLLIKNQKKVITREMILSQAWDFAAIVDTSIINTHMKNLRKKLHTEKIQTIRGAGYKMDE               Lmo phoP
152 EYEVLLYLAKRRGKIVSRDNLMMGIWGYTEL-DSRVLDNHIKNIRKKLPSLPLKTIVGRGYQIEGT              Ssu
```

Figure 25: Sequence alignment of RR13 of *S. pyogenes* strain SF370 with similar response regulators of other bacterial species. Spy = *S. pyogenes*; *Bce* = *Bacillus cereus*; *Fear* = *Enterococcus faecalis*; *Lmo* = *Listeria monocytogenes;* Ssu = *Streptococcus suis*. Conserved residues are indicated in red.

#### 2.6.2.1.2. Structural analysis of the histidine kinase HK13

The histidine kinase HK13 is composed of 463 amino acids and can be grouped into the HK2a subfamily of histidine kinases, to which EnvZ as one of the most thoroughly characterized histidine kinase belongs (57, 211). Prediction by hydrophobicity scans revealed the presence of two membrane-spanning regions, the first one ranging amino acid 7 to 28, and the second one from amino acid 154 to 173. Therefore, HK13 harbours an extracellular, 126 amino acid long sensing domain starting at position 29 (alanine) and ending at position 153 (aspartate). In order to identify putative signal molecules, the sequence of this sensing domain was compared with entries of protein databases; however, no similarities were found.

Sequence analysis of HK13 identified four out of five for histidine kinases characteristic homology boxes (see section 1.2 and Tab. 9). The H-Box harbouring the phospho-accepting histidine (position 252) is located from amino acid 243 to 261. The X-region ranges from

amino acid 285 to 305, and secondary structure prediction could confirm the characteristic α-helical form (82) within this region. The conserved asparagine is located at position 362; the N-Box itself is ranging from amino acid 352 through 371. One of the homology boxes that could not be identified is the D-Box, which is usually located between the N- and F-Box and contains a conserved aspartate as part of the nucleotide-binding domain. The predicted location of this residue is position 380; it is lacking in HK13 of strain SF370, as well as in the other sequenced *S. pyogenes* genomes. Fig. 27 depicts all conserved residues of the D-box as grey shades, however this signature sequence has not been named due to the absent aspartate. The F-Box, which succeeds the D-Box, is present again; the conserved asparagine after which this region has been named is positioned at amino acid 405 within HK13. Ranging from amino acid 420 through 426, the G-Box involved in ATP-binding is located.

Table 9: Positions of homology boxes found in HK13 with and conserved residues.

| **Homology box / region** | **Amino acid positions** | **Conserved residue and position** |
|---|---|---|
| H-Box | 243 - 261 | Histidine 252 |
| X-Region | 285 - 305 | none in HK 2a |
| N-Box | 352 - 371 | Asparagine 362 |
| D-Box | absent | |
| F-Box | 402 - 409 | Phenylalanine 405 |
| G-Box | 420 - 426 | none |

Searches for histidine kinases sharing sequence homologies with HK13 revealed a number of similar kinases, of which the best characterized one is the HK VanS of *E. faecalis* strain V583 (accession number Q47745). VanS is the corresponding sensor to the response regulator VanR mentioned in 2.6.2.1.1., and is involved in the sensing process of vancomycin. A similar kinase named VncS involved in adaptive responses to vancomycin as well is described in *S. pneumoniae* (163, 184). Besides these two histidine kinases, HK13 shares similarities to a yet uncharacterized kinase in *Streptococcus suis* (Acc. No. ZP_00333076), and *Bacillus cereus* (Acc. No. NP_977908). An alignment of HK13 with all named histidine kinases is illustrated in Fig. 27. Transmembrane domains, as well as homology boxes with their conserved residues are indicated in shades and red. Interestingly, all similar histidine kinases also lack the in HK13 absent D-Box. Additionally, the extracellular sensing domain located in between the two transmembrane helices does not share any sequence homologies between the compared kinases, as seen by long stretches of non-alignable amino acids.

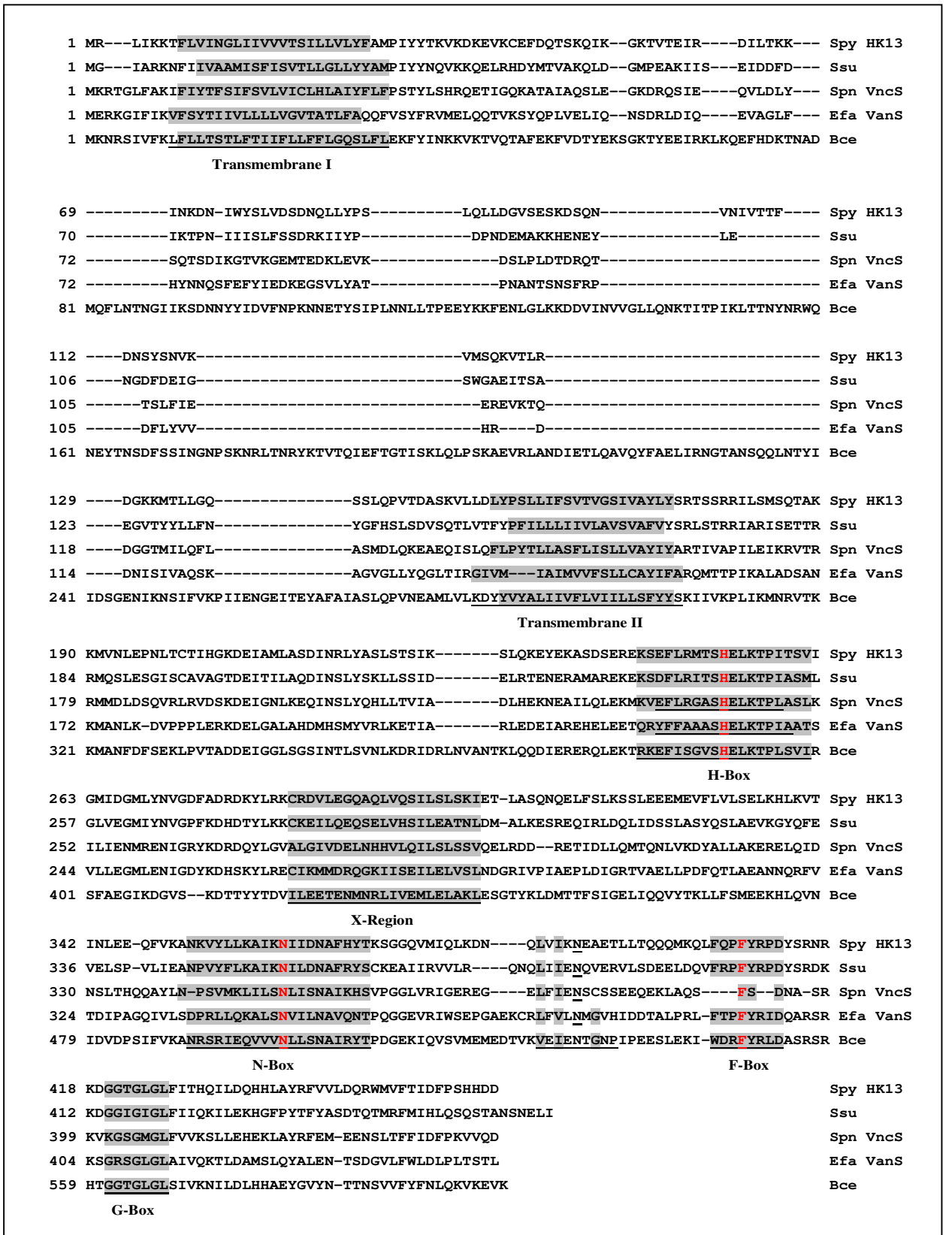



Figure 27: Sequence alignment of HK13 of *S. pyogenes* M1 with homologue histidine kinases from other bacterial species. Spy = *S. pyogenes*; Ssu = *Streptococcus suis*; Spn = *Streptococcus pneumoniae*; Efa = *Enterococcus faecalis*; Bce = *Bacillus cereus*. Conserved residues are indicated in red, signature sequences and transmembrane helices are shaded. The missing D-Box is indicated between the N- and F-Box.

### 2.6.2.2. Chromosomal neighbourhood of the TCS13

TCS13 was chosen for mutagenesis studies due to its interesting chromosomal neighbourhood, which comprises a number of well-characterized streptococcal virulence genes. A more detailed overview of the chromosomal neighbourhood, including putative transcriptional terminators is given in Fig. 28. In the other so far sequenced *S. pyogenes* genomes, the neighbourhood of this two-component system is nearly identical. Upstream of *rr*13 lies an ABC transporter, which shows homologies to a transporter with unknown functions from *Streptococcus cristatus*. Downstream of the histidine kinase *hk13*, the gene *isp* is located, which encodes the immunogenic secreted protein, and probably lies within the same operon as TCS13. The biological function of Isp is yet unknown, however McIver *et al.* (148) have demonstrated that inactivation of the gene had no effect on the transcription of M-protein. Nevertheless, it is expressed and exposed to the host during infection, since serum isolated from a patient suffering from streptococcal infection recognized forms of this protein. Additional analysis of Isp using protein family searches (PFAM) revealed the presence of a CHAP (cysteine, histidine-dependent amidohydrolases/peptidases) domain within this protein. These domains are found in many uncharacterized proteins for which a function in peptidoglycan hydrolysis has been proposed (13). Consequently, Isp might have a similar function in *S. pyogenes*.

Downstream of *isp*, the gene for the multiple gene activator Mga is located, which influences expression of a number of virulence factors (see section 1.3.2.1). Downstream of *mga*, *sic*, as well as an uncharacterized open reading frame of 84 amino acids, and *scpA*, encoding C5a peptidase precursor, are located.

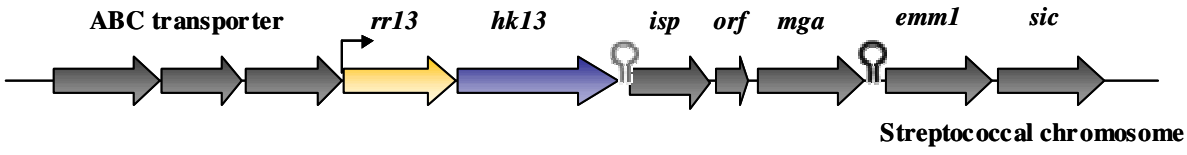



Figure 28: Chromosomal neighbourhood of *tcs13* (response regulator rr13 in yellow, histidine kinase hk13 in blue). *Isp* refers to the structural gene of immuno-secreted protein, *mga* encodes the single regulator Mga, *emm1* the M1 protein, *orf* represents an uncharacterized open reading frame, and *sic* the streptococcal inhibitor of complement. Downstream of tcs13 three structural genes encoding an ABC transporter are located. Putative transcriptional starts are indicated as arrows, terminators as loops.

### 2.6.2.3. Growth characteristics of the TCS13 mutant

In regular culture broth, the TCS13 mutant did not show any altered growth rate (see section 2.4.), and therefore met the requirements needed for further studies. However, the TCS13 mutant showed different sedimentation behaviour in comparison to the wildtype. In mid-exponential to stationary phase, the wildtype showed strong aggregative behaviour, however the mutant did not (Fig. 29). This phenomenon has already been observed with the TCS07 mutant, where it was related to the lack of M protein expression on the bacterial surface (2.6.1.5.3). Therefore, this observation might again be directly linked to a reduced expression of this virulence factor on the surface of the TCS13 mutant. Results related to transcription of *emm1* and presence of M-protein on the surface of the TCS13 mutant will be presented in section 2.6.3.7.1.

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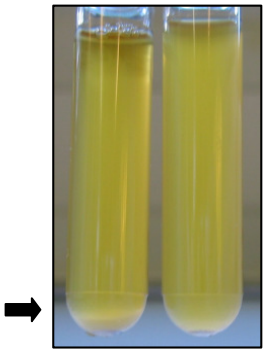



Figure 29: Sedimentation behaviour of KTL3 and its isogenic mutant ΔTCS13. An arrow indicates sedimented wildtype bacteria.

### 2.6.2.4. Contribution of the TCS13 to bacterial virulence in a mouse model of infection

Although the role of the TCS13 in resistance to killing by phagocytic cells has already been demonstrated within the Lancefield Bactericidal Assay, its contribution to bacterial virulence during *in vivo* infection remains unknown. Using two different mouse models of infection (213), the contribution of TCS13 to streptococcal virulence was evaluated in terms of (i) survival times of infected mice; (ii) bacterial dissemination to systemic organs; (iii) resistance to phagocytosis by PMNs and macrophages.

#### 2.6.2.4.1. Survival time of infected animals

In a first approach, the survival times of mice subcutaneously infected with $2.5 \times 10^8$ cfu of either wildtype or mutant was monitored over a period of seven days. Results depicted in Fig. 30 show that while 100 % of animals infected with the wildtype strain died between day 2 and three postinoculation, 80 % of mice infected with the mutant survived until day 7. The

extended survival of ΔTCS13-infected animals suggests a loss of virulence associated with the knock-out of this regulatory system. However, the severeness of the skin lesions was comparable in both groups of mice, indicating a similar capacity of wildtype and mutant to survive and proliferate at the local infection site.

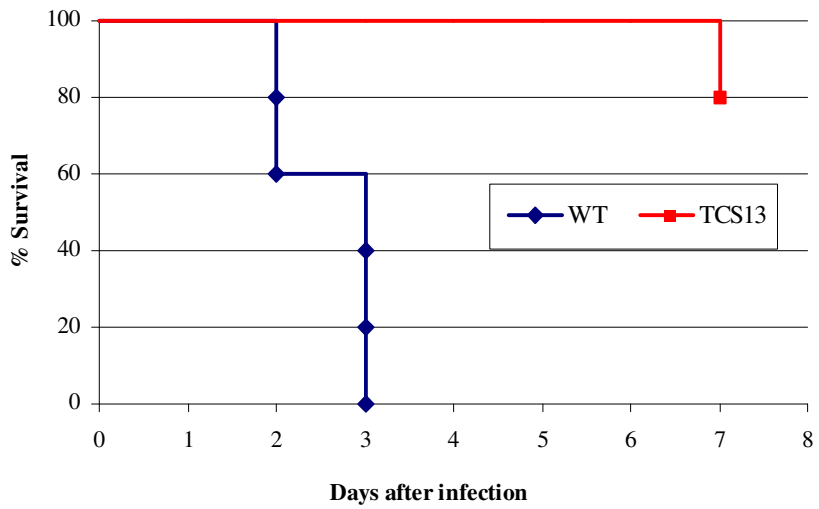



Figure 30: Kaplan-Meyer plot showing the survival times of mice subcutaneously infected with $2.5 \times 10^8$ cfu of either KTL3 wildtype or ΔTCS13.

### 2.6.2.4.2. Dissemination of bacteria from local infection foci to systemic organs and bloodstream.

The possibility that an impaired ability of the mutant to spread from the local site of infection to the bloodstream and systemic organs might be responsible for the increased survival observed in ΔTCS13-infected mice was evaluated. Mice were infected subcutaneously with either wildtype or mutant, and the bacterial load at 24, 48, and 72 hours postinfection in blood and the whole systemic organs was determined. Because of the short survival times, bacterial loads in mice infected with the wildtype were only determined 24 hours postinfection (Fig. 31). At this time of infection, between groups of mice infected with wildtype and mutant significant differences were found in the number of bacteria per organ. Thus, while wildtype-infected animals exhibited 5.6 $\log_{10}$ bacteria in liver, 5.8 $\log_{10}$ in spleen, and 5.5 $\log_{10}$ in blood, bacterial counts in ΔTCS13-infected mice were lower with only 3.0 $\log_{10}$ bacteria detected in the liver, 1.57 $\log_{10}$ in spleen, and none in the blood. The number of bacteria was stabilized between 24 and 48 hours postinfection with a sharp decrease observed at 72 hours

(Fig. 31). When bacterial counts in the skin of mice were determined, no significant differences were found between the wildtype- and mutant-infected animals.

These results suggest that the TCS13 mutant is either impaired in the ability to spread from the local site of infection to the bloodstream and systemic organs, or it is more readily killed than the parental strain once it reaches the bloodstream; both possibilities can explain the lower bacterial count found in blood and organs of mice infected with ΔTCS13. The regulatory system seems does not play a role in the bacterial capability to survive and proliferate at the local infection site since the severity of the lesions were comparable for both, wildtype and parental strain.

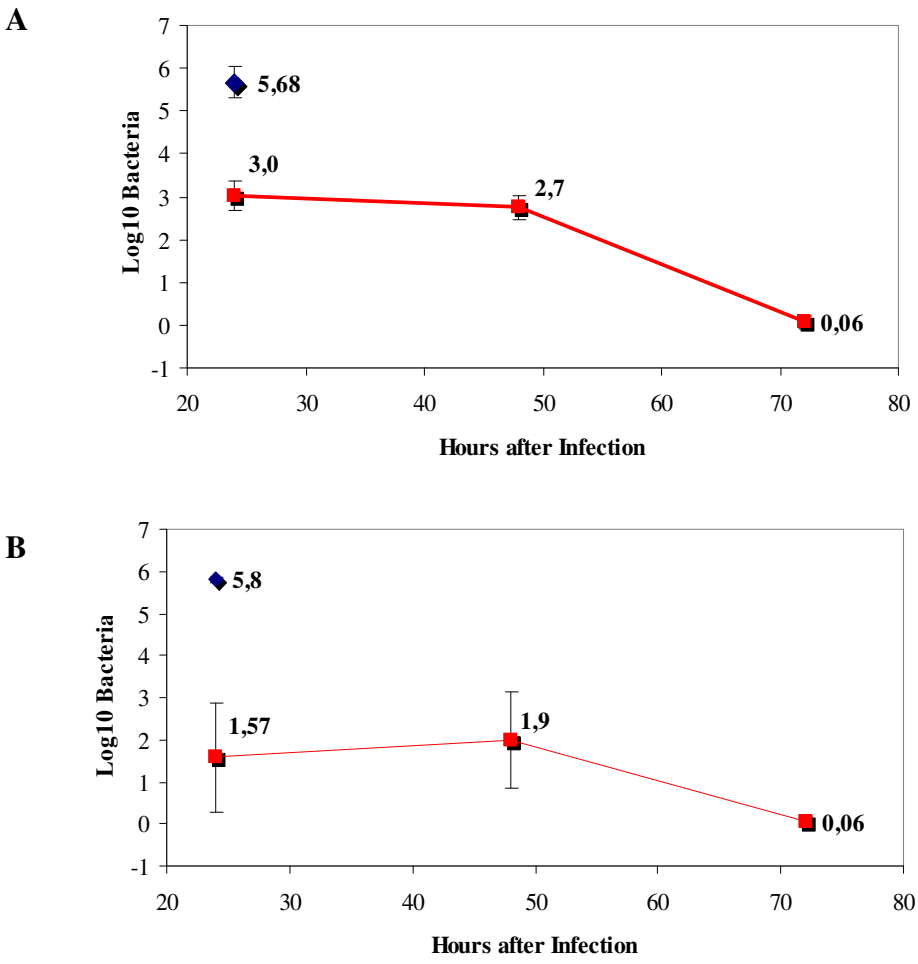



Figure 31: Distribution of KTL3 wildtype and ΔTCS13 bacteria in mouse organs at 24, 48, and 72 hours after subcutaneous infection with $2.5 \times 10^8$ cfu. KTL3 is displayed in blue, ΔTCS13 in red. (A) Bacteria isolated from liver. (B) Bacteria isolated from spleen. Bacterial loads in the blood of infected mice are not shown.

#### 2.6.2.5. *In vivo* phagocytosis assay

The ability of either the wildtype, or the ΔTCS13 strain to resist phagocytosis by PMNs and macrophages was evaluated using an *in vivo* phagocytosis assay developed by Goldmann *et al*. (78). This assay is based on the introduction of bacteria into the peritoneal cavity of mice and allows the evaluation of phagocytosis by either PMNs, which accumulate at the site of infection after 2-4 hours, or macrophages, which are present in the peritoneum ready to phagocyte bacteria immediately after inoculation.

##### 2.6.2.5.1. Bacterial uptake by murine macrophages

First, bacterial uptake by murine macrophages was investigated for KTL3 and ΔTCS13. For that purpose, mice were intraperitoneally infected with $5 \times 10^7$ cfu of green-labelled wildtype- or mutant bacteria, left for 30 minutes for the phagocytic process to take place, and macrophages were isolated thereafter by performing peritoneal lavages. They were specifically labelled with a red-conjugated antibody and analysed by flow-cytometry (FACS). Double-positives, i.e. green and red fluorescent cells, represented macrophages associated with bacteria.

The results displayed in Fig. 32 show the phagocytic activity of macrophages towards wildtype and ΔTCS13. Groups of mice infected with non-labelled bacteria were used as control (Fig. 32A and C). The percentage of macrophages associated with KTL3 and ΔTCS13 is shown in the upper right square of Fig. 32B and D. 95.3 and 98 % of all isolated macrophages were associated with wildtype and mutant bacteria, respectively, indicating a strong phagocytic activity of these cells towards both microorganisms.

FACS analysis has limitations, since macrophage-associated bacteria cannot be distinguished from intracellular-located ones. To distinguish between these two states, intraperitoneal infection experiments with non-labelled bacteria were performed and after isolation of macrophages, extracellularly attached bacteria were killed by gentamycin treatment. Cells were seeded onto coverslips, fixed, and for differentiation between intra- (red) and extracellular (green) bacteria, double immune fluorescence (DIF) was performed. Photographs displayed in Fig. 32 show wildtype (32E) and ΔTCS13 (32F) located mostly inside of macrophages, as seen by red colour. Due to the fluorescent filter, macrophages are only visible as shades when associated with bacteria. No differences can be detected between wildtype and mutant, indicating a similar ability of macrophages to phagocyte both strains.

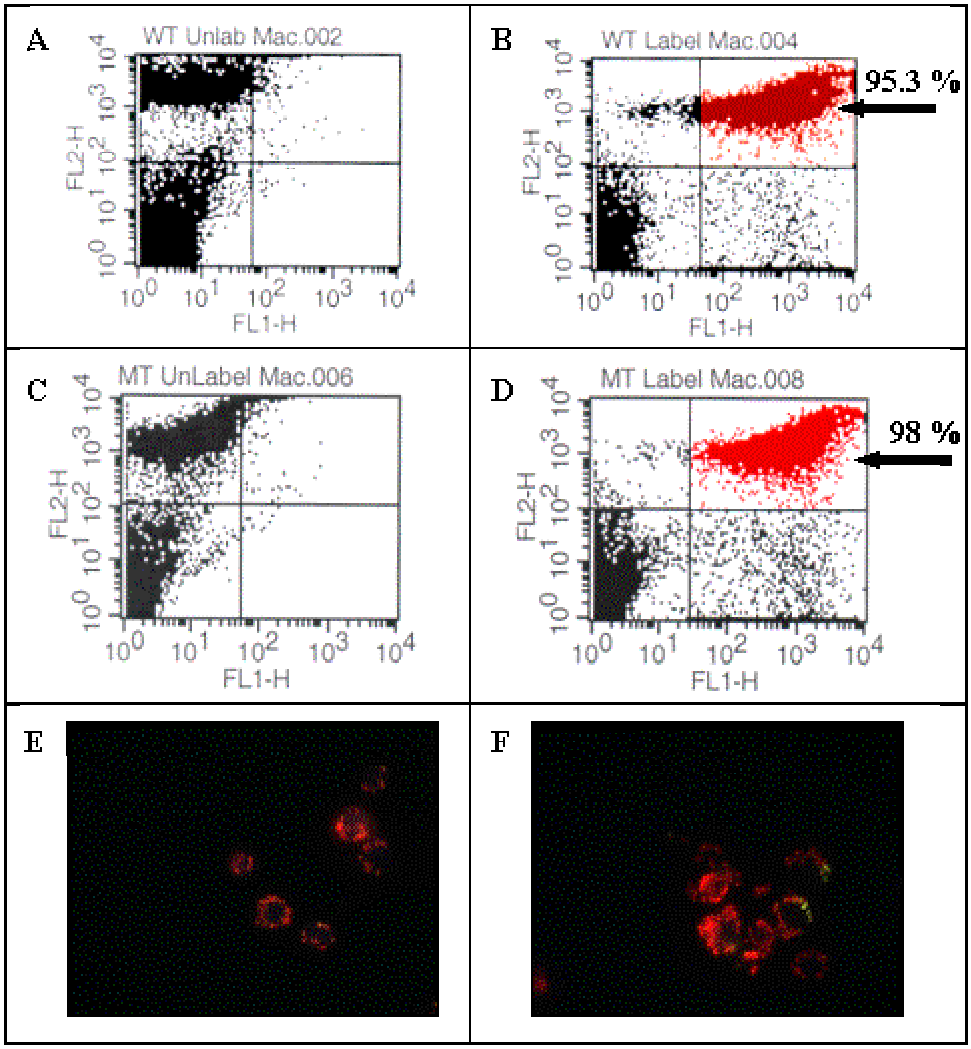



Figure 32. A-D: FACS analysis of red-fluorescent-labelled macrophages associated with green-fluorescent-labelled KTL3 and ΔTCS13. FL1-H (abcissa): relative green fluorescence (bacteria); FL2-H (vertical axis): relative red fluorescence (macrophages). Lower left square within each dotplot shows the non-macrophage population, the upper left square shows the labelled macrophage population, the lower right square corresponds to green fluorescent bacteria, and upper right square shows macrophages associated with wildtype (B) or mutant (D). Macrophages associated with unlabeled wildtype (A) or mutant (C) were used as control. E-F: Double-immune fluorescent photograph of wildtype (E) and ΔTCS13 (F) associated with macrophages. Extracellular bacteria appear in green, intracellular bacteria in red.

#### 2.6.2.5.2. Bacterial uptake by murine PMNs

The phagocytic activity of the second major phagocytic cell population (PMNs) towards wildtype and ΔTCS13 was investigated. For this purpose, mice were infected intraperitoneally with $5 \times 10^7$ cfu of either green-labelled wildtype or mutant, and left for two hours, allowing infiltration of PMNs from the blood to the infection foci and phagocytosis of bacteria. PMNs were isolated by peritoneal lavage and specifically labelled with a red-fluorescent antibody. Subsequent FACS analysis was performed for quantifying the percentage of PMNs associated with either wildtype or mutant, represented by double-positive (green and red) cells.

The results in Fig. 33 show the phagocytic activity of murine PMNs towards wildtype and ΔTCS13. Again, groups of mice infected with non-labelled bacteria were used as control (Fig. 33A and C). The upper right square of Fig. 33B and D represents the percentage of PMNs associated with wildtype and ΔTCS13, respectively, and demonstrates that only 2.47 % of the wildtype, and 2.59 % of the mutant were phagocytosed. This low uptake could either be due to (i) a low phagocytic activity of PMNs towards both strains, or (ii) high phagocytic activity of macrophages, resulting in low amounts of bacteria remaining in the peritoneum after two hours. To further elucidate this possibility, mice were depleted of macrophages by treatment with carrageenan two days prior to infection, which additionally led to a significant increase of circulating PMNs. Carrageenan-treated mice were infected intraperitoneally with $5 \times 10^7$ cfu of either wildtype or ΔTCS13, and PMNs were isolated by peritoneal lavage after 1 hour of infection. Extracellularly attached bacteria were killed by gentamycin treatment and PMNs were seeded onto poly-L-lysine coated coverslips to allow attachment of these usually non-adherent cells. PMNs were fixed, and immune staining was performed for detection of intra- and extracellular bacteria. Photographs of PMNs associated with red-stained intracellularly located wildtype and mutant bacteria are displayed in Fig. 33E (wildtype) and 33F (ΔTCS13), and show that most bacteria were situated intracellularly, independently of wildtype or mutant. Additionally, almost all PMNs were associated with bacteria. These results indicate that (i) results obtained before showing low amounts of bacteria associated with PMNs (Fig 33B and 33D) are due to high phagocytic activity of macrophages, (ii) there are no differences in the ability of PMNs to phagocyte either wildtype or ΔTCS13.

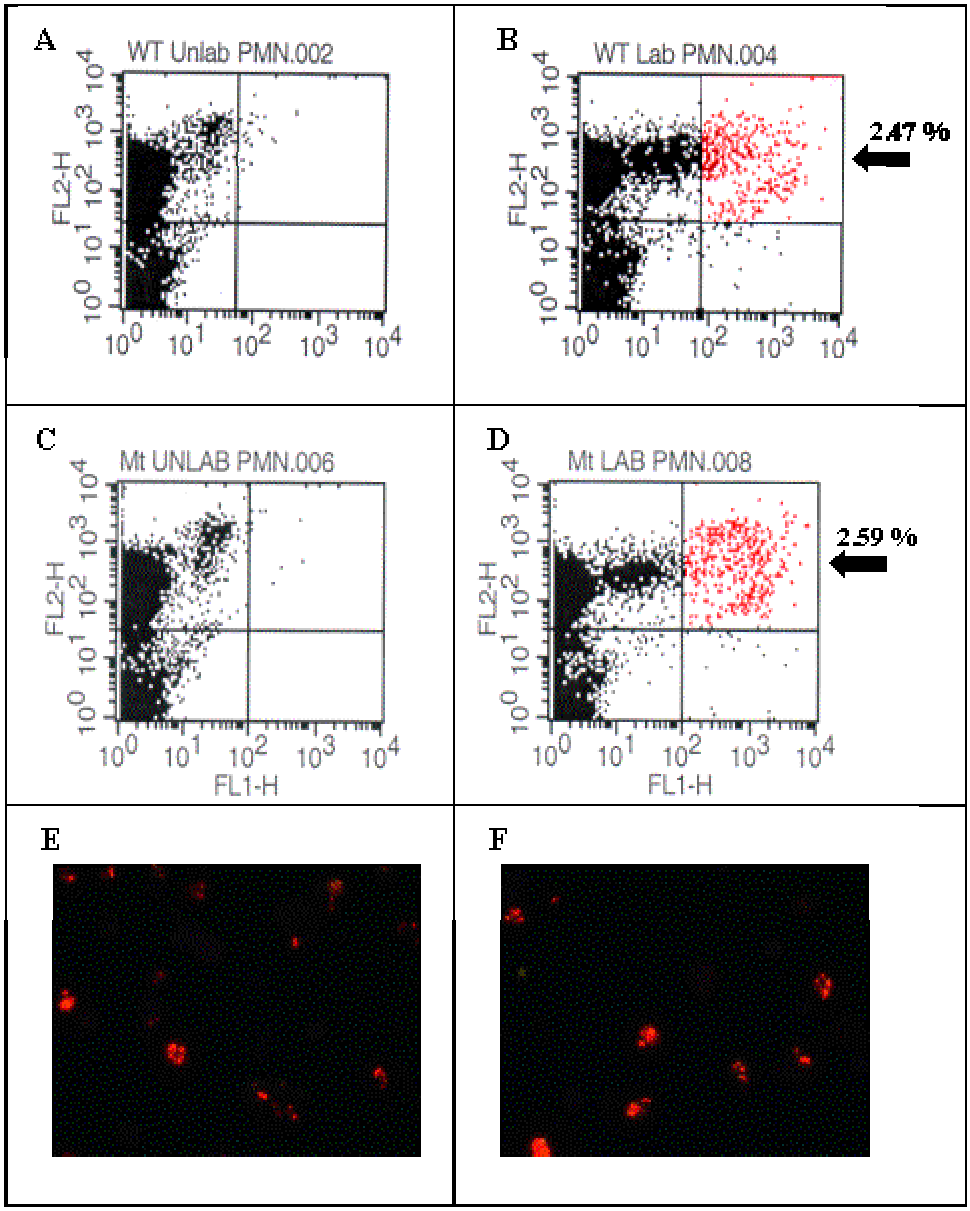



Figure 33. A-D: FACS analysis of red-fluorescent-labelled PMNs associated with green-fluorescent-labelled KTL3 and ΔTCS13. FL1-H (abcissa): relative green fluorescence (bacteria); FL2-H (vertical axis): relative red fluorescence (PMNs). Lower left square within each dotplot shows the non-PMN population, the upper left square shows the labelled PMN population, the lower right square corresponds to green fluorescent bacteria, and upper right square shows PMNs associated with wildtype (B) or mutant (D). PMNs associated with unlabeled wildtype (A) or mutant (C) were used as control. E-F: Double-immune fluorescent photograph of wildtype (E) and ΔTCS13 (F) associated with PMNs. Intracellular bacteria are displayed in red.

#### 2.6.2.5.3. Macrophage- and PMN killing assay

The assay for bacterial uptake does not allow a differentiation between internalized live or dead bacteria, since dead bacteria and bacterial debris can still be detected by fluorescence. Since no differences could be observed between wildtype and mutant in the ability to resist uptake by phagocytic cells, the question remains of whether the attenuation of the wildtype and mutant strain seen in the animal model was due to a higher killing of these microorganisms after phagocytosis. To address this point, mice were intraperitoneally infected with non-labelled wildtype or mutant bacteria, and macrophages were isolated after 30 minutes. For PMNs, carrageenan-treated animals were infected with wildtype or mutant, left for one hour, and PMNs were isolated by peritoneal lavage. Macrophages and PMNs were separately seeded into cell culture wells and treated with gentamycin for killing of extracellular located wildtype and mutant bacteria. For determining the amount of intracellularly located, live bacteria at time-point zero, half the wells containing PMNs and macrophages were treated with Triton-X-100, resulting in lysis of cells and release of intracellular bacteria, which were subsequently plated onto blood agar. The other wells were incubated for additional two hours, treated as described above, and the amount of remaining intracellular live wildtype and ΔTCS13 were determined by plating onto blood agar.

Results displayed in Figure 34 show the percentage of viable wildtype and mutant bacteria isolated from either macrophages (34A) or PMNs (34B) after two hours of incubation and show that there are no differences in the killing rate of wildtype and mutant mediated by macrophages. Both strains are reduced by about 88 %, corroborating with the ability of macrophages to kill phagocytosed *S. pyogenes*. On the other hand, there is a clear difference seen in the ability of wildtype and mutant to resist killing by PMNs, the killing rate towards the ΔTCS13 strain is about 30 % higher than seen in the wildtype strain, resulting in nearly complete elimination of the mutant.

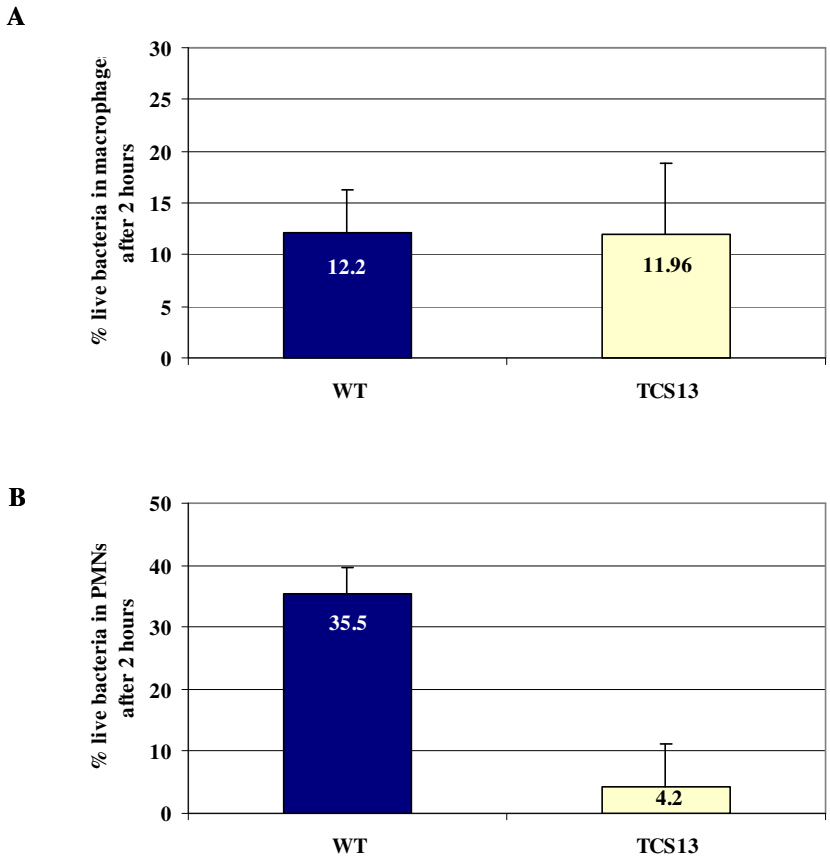



Figure 34: Survival of wildtype and ΔTCS13 in murine macrophages (A) and PMNs (B) after two hours. Results are shown as percentage of the initial number of intracellularly located bacteria at time-point zero, which refers to the bacterial number after gentamycin treatment.

These results show that (i) macrophages are capable of efficiently killing wildtype and ΔTCS13 strain to a similar degree, and (ii) that in contrast to the wildtype, the ΔTCS13 strain is nearly completely eliminated by PMNs. Consequently, it seems that the TCS13 confers high resistance against phagocytic killing, enabling bacteria a superior intracellular survival, as recently shown for human PMNs (219).

### 2.6.2.6. Transcription analysis of virulence factors

Results obtained from the Lancefield Bactericidal Assay (2.5.) and the Macrophage- / PMN killing assay (2.6.2.5.3.) indicate an altered resistance to killing after phagocytosis, since mutant bacteria are more readily eliminated. A number of different streptococcal surface proteins have been shown to be involved in resistance to phagocytosis and killing such as the M protein and SIC (see section 1.1.2.).

For the identification of the underlying molecular factors responsible for the elimination of mutant bacteria, transcription levels of two main antiphagocytic proteins – M1 protein and streptococcal inhibitor of complement (SIC) - were compared between wildtype and ΔTCS13 mutant. Results shown in Fig. 35 demonstrate a strong transcription of both virulence factors in the wildtype strain. Transcriptional analysis of these factors in the mutant, however, showed comparable transcript levels. Therefore, both virulence factors are transcribed at the same rate, demonstrating that differences seen in the Lancefield Assay are not due to lower amounts of transcription of these genes in the regulatory mutant.

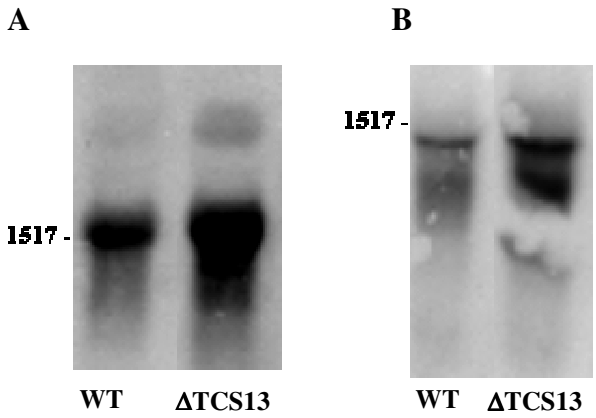



Figure 35: Northern Blot analysis of broth-derived KTL3 and ΔTCS13. (A) Detection of *emm1* transcript (M protein). (B) Detection of *sic* transcript (SIC).

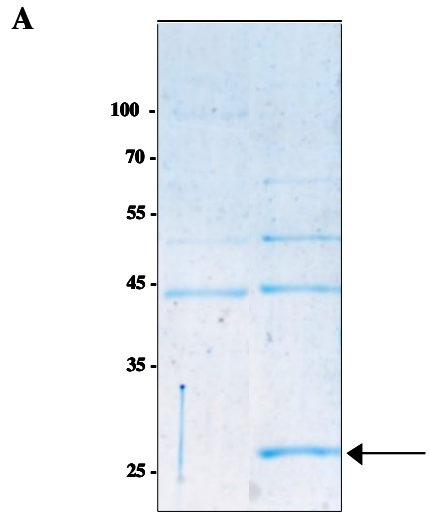
### 2.6.2.7. Expression and processing of virulence factors

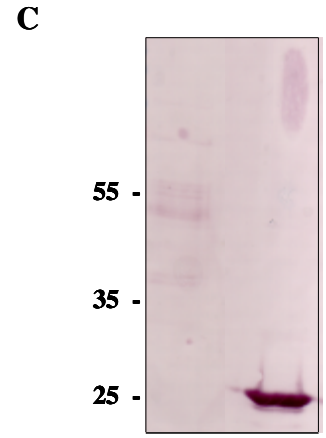
Transcriptional analysis of known main antiphagocytic streptococcal factors did not show differences between wildtype and mutant and could therefore not explain the differences seen in the Lancefield Assay. Since regulation at the posttranscriptional- and modifications at the postranslational level are frequently found for streptococcal surface proteins (e.g. by SpeB, 1.1.2.5.) analysis of protein amounts and activity were performed in parallel. The hyaluronic acid capsule as non-proteinacious antiphagocytic factor was included.

#### 2.6.3.7.1. M1 protein

In order to detect possible differences in the presence of streptococcal surface proteins between the wildtype and regulatory mutants, protein lysates containing cell wall fractions and cytosolic proteins, as well as supernatants containing secreted streptococcal proteins were

separated by 1-dimensional gelelectrophoresis. No differences could be seen between cell wall / cytosolic fraction of wildtype and ΔTCS13 (data not shown), however in TCA-precipitated supernatants a ≈ 28 kDa sized protein was observed in the regulatory mutant (Fig. 36A) that was absent from the culture supernatant of the wildtype. Maldi-TOF analysis of this 28 kDa protein revealed this protein to be the N-terminal fraction of the M-protein (reliability-score: 93), matching distinct peptides ranging from amino acid 31 to 180 of the M protein precursor (Acc. Nr. AAN46657) (Fig. 36B). Western Blot analysis using KTL3 M1-specific polyclonal antibodies confirmed this fraction to be M protein (Fig. 36C). These results demonstrate that a processing of the M protein in the ΔTCS13 strain occurs, releasing the N-terminal part of this important virulence factor from the streptococcal surface.




**B**

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  1 TASVAVALTV LGAGFANQTE VKANGDGNPR EVIEDLAANN PAIQNIRLRH ETKDLKARLE
 61 NAMEVAGRDF KRAEELEKAK QALEDQRKDL ETKLKELQQD YDLAKESTSW DRQRLEKELE
121 EKKEALELAI DQASRDYHRA TALEKELEEK KKALELAIDQ ASQDYNRANV LEKELETITR
181 EQEINRNLLG NAKLELDQLS
```

Figure 36: (A) Coomassie stain of KTL3 (lane 1) and ΔTCS13 (lane 2) precipitated supernatants. Arrow indicated the truncated M protein. (B) Maldi-TOF analysis of the truncated M protein. Matched peptides are shown in red. (C) Western Blot analysis of the precipitated supernatant using an M1-specific polyclonal antibody. Lane 1: wildtype, lane 2: ΔTCS13.

Sequence comparisons of the released M1 protein with published structural analyses of the M6 protein (68), revealed the site of truncation within the variable B-repeats of the protein (Fig. 37).

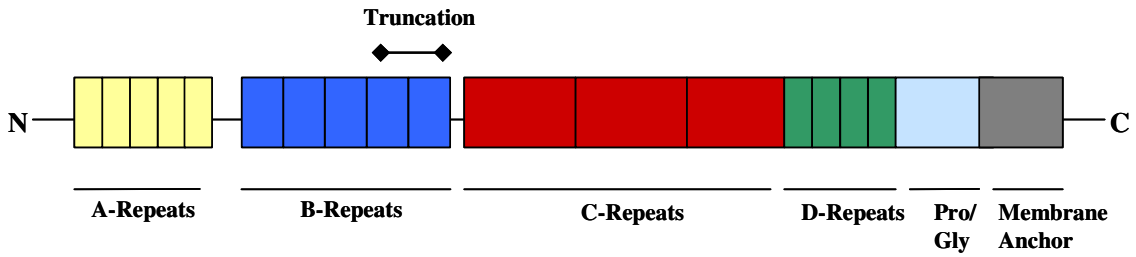


Figure 37: Schematic overview of the M protein as already shown in the introduction (1.1.2.2.). The area of truncation, resulting in release of the 28 kDa fragment is indicated.

The A- and B repeats of the M protein mediate fibrinogen binding (17, 227), an attribute that leads to obstruction of complement binding to streptococcal cell wall structures and therefore protection against uptake by phagocytic cells (227). Truncation of the M protein within the B-repeats could result in loss of the fibrinogen-binding activity, thus explaining increased killing within the Lancefield Bactericidal Assay, as well as the attenuation of the mutant in the mouse model of infection. Therefore, the M protein's fibrinogen binding capability of ΔTCS13 was more closely evaluated. For this purpose, fibrinogen binding of the truncated 28 kDa fragment was investigated by Western analysis. Blotted 28-kDa fragments were incubated with human fibrinogen, subsequently followed by detection using an anti-fibrinogen antibody. Results shown in Fig. 38 show a strong signal from the 28-kDa fragments, as well as from the recombinant M protein, which served as positive control. A negative control, incubating the 28-kDa fragments with the applied antibodies, gave no signal (data not shown). These results clearly show the fibrinogen binding capability of the truncated M protein.

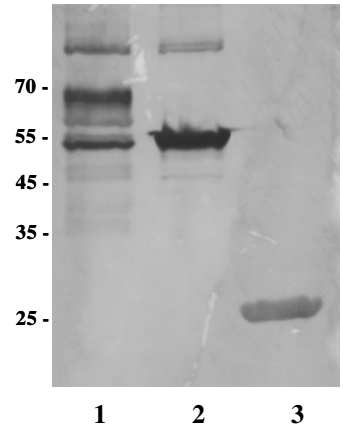


Figure 38: Binding of immobilized M protein to human fibrinogen. 1. Human fibrinogen (positive control); 2. Recombinant M1 protein (positive control); 3. Truncated M protein from ΔTCS13.

Loss of the N-terminal, fibrinogen-binding region of the M protein would ultimately lead to the inability of the ΔTCS13 strain to bind this plasma protein. Consequently, it was investigated whether and to what degree whole mutant streptococci were able to bind to radiolabelled fibrinogen. Results presented in Fig. 39 show the binding capability of KTL3 and ΔTCS13 as percentage of the fibrinogen used in this assay. The wildtype showed a high fibrinogen binding ability; it bound 84.19 % of all applied fibrinogen, which corresponds to approximately 505 ng. In contrast, ΔTCS13 merely bound 12.56 % of the fibrinogen, corresponding to ≈ 75 ng, showing that the regulatory mutant bound almost 7 × less labelled fibrinogen that the wildtype. These results stress the importance of the M protein in fibrinogen binding and correlate the truncation of this protein in the TCS13 mutant with a loss of binding capability.

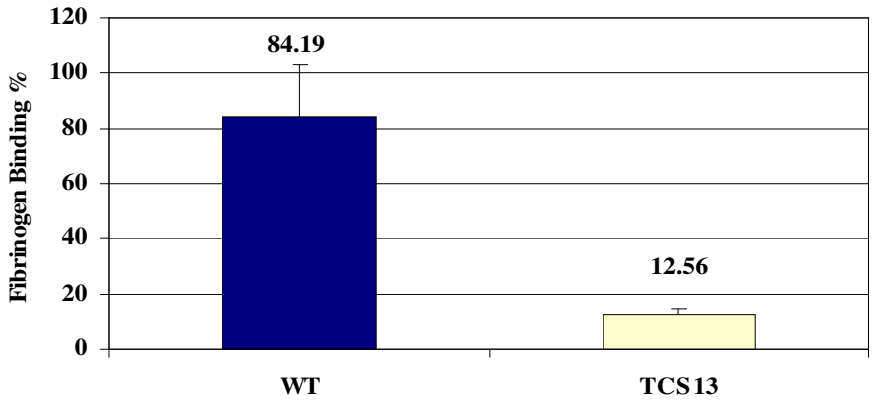


Figure 39: Binding of radiolabelled fibrinogen to KTL3 and ΔTCS13. Results represent the mean of three independent experiments and are presented as percentage of all fibrinogen used in this assay.

If a cleavage of the M protein from the surface of ΔTCS13 occurs, the approximately 27 kDa sized C-terminal part, composed of the last B- and the C-repeats (Fig. 37) should be detectable on the surface of the *Streptococcus* Acidic extractions of the M protein from the streptococcal surface were used for Western blot with polyclonal anti-M1 antiserum, raised against the whole protein. Maybe due to the high inspecificity and binding of the antibody to a number of streptococcal proteins under denaturing conditions, no band specific for a truncated M protein could be identified (data not shown). Another possibility to detect surface-located M1 protein is direct microscopic analysis using a gold-labelled M1 antibody. Since this method is performed under native conditions, the cross-reactivity of the antibody with other streptococcal proteins under denaturing conditions is minimized. Therefore, broth-grown bacteria were incubated with purified M1 antibody, and visualized using field emission scanning electron microscopy (FESEM). Again, since the M1 antibody recognizes the whole M1 protein, the remaining cleaved fragment located on the bacterial surface should be detectable. Figure 40A shows wildtype bacteria incubated with anti-M1 antibody. On the surface, a high amount of M protein is detectable, as seen by white dots. In some regions, these dots appear in chains, which is probably due to multiple binding of the polyclonal antibody to different region of the surface protein. Figure 40B, on the other hand shows the ΔTCS13 strain. Unexpectedly, less M protein seem to be localized on its surface. However, still more protein is visible than in the negative control, which in this case is the M-negative strain KTL3ΔTCS07 (Fig. 21 page 44). The appearance of less M protein on the surface of ΔTCS13 could be caused by: (i) comparable expression of the M protein, but instability of the C-terminal, surface-located part, (ii) lower expression of M protein in the mutant, or (iii) comparable expression, but a reduced number of binding sites for the polyclonal antibody, due to the shorter length of surface-located M protein. However, the C-terminal part of the protein is still located on the surface of ΔTCS13, and consequently the release of the 28 kDa fragment is with high probability due to a protease activity. The cysteine protease SpeB is known to mediate cleavage of the M protein from the streptococcal surface, and therefore its activity in wildtype and mutant was investigated in section 2.6.2.7.2.

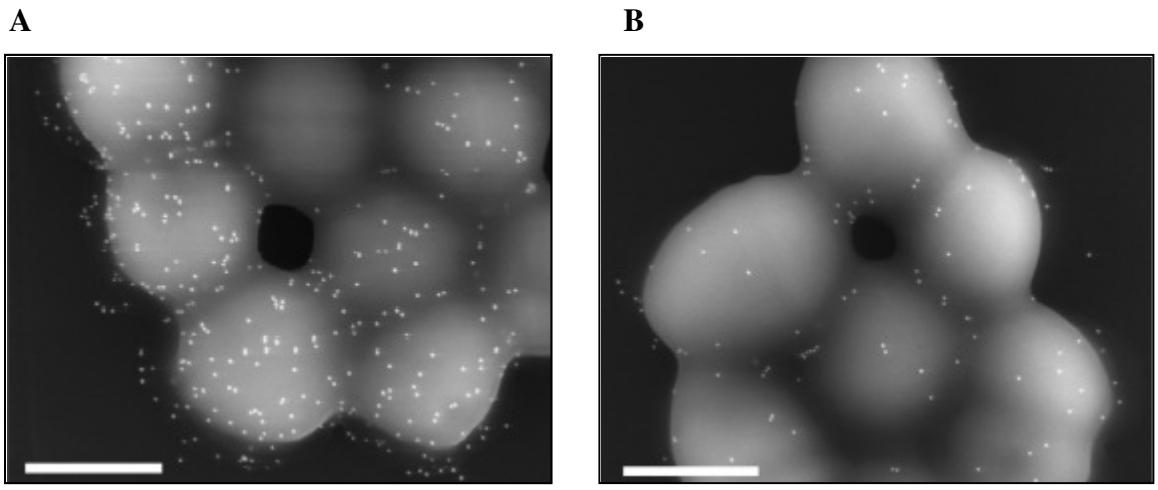


Figure 40: FESEM picture of KTL3 (A) and ΔTCS13 (B), with gold-labelled anti-M1 antibody. Bar corresponds to 0.5 µm.

#### 2.6.2.7.2. M protein-directed proteolytic activity

It has been described that the cysteine protease SpeB leads to degradation of the M protein and is mainly upregulated during late-logarithmic, to stationary growth phase (137). Proteolytic activity of SpeB in culture supernatants can be determined on the basis of degradation of azocasein, which can be photometrically detected. Analysis of the proteolytic activity of SpeB in culture supernatants revealed no differences between KTL3 and ΔTCS13, as seen in Figure 41. Wildtype supernatants exhibited an average absorbance at 366 nm of –0.0455; the absorbance of supernatants derived from the mutant was with –0.0625 almost equal to the values obtained from the parental strain. Negative optical densities of both strains indicated no SpeB activity. In contrast, supernatants of the positive control A5448 showed an absorbance of 0.49. When cultures were grown in the presence of cysteine protease inhibitor and subjected to activity measurement, the absorbance of the positive control dropped to -0.053, showing the efficacy of the inhibitor and specificity of this assay for SpeB. These results show that in both, wildtype and ΔTCS13 mutant, the SpeB activity is low and therefore this protease is not the responsible factor leading to the cleavage of the M protein from the surface of ΔTCS13.

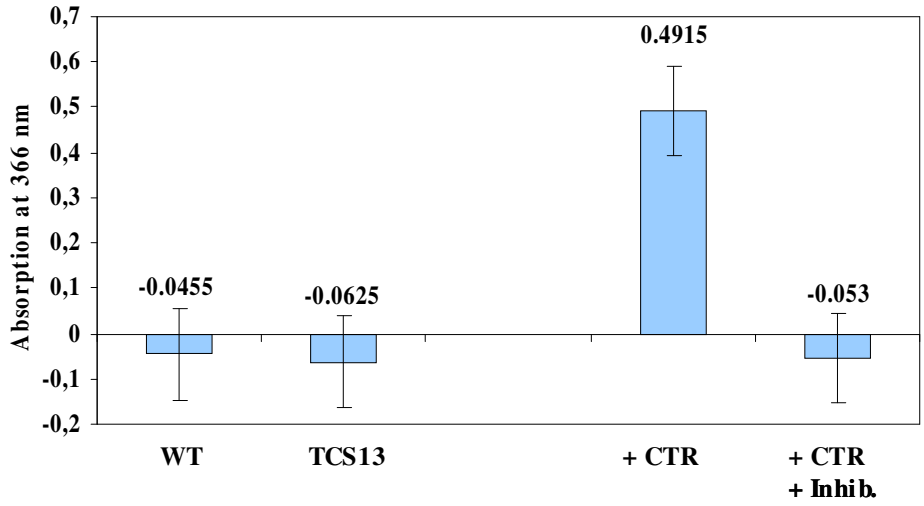


Figure 41: Analysis of proteolytic activity in culture supernatants from KTL3, ΔTCS13, and strain A5448 as positive control grown in THY medium after activation of SpeB by a reducing agent. Absorbance was measured at 366 nm.

To further prove the hypothesis that not SpeB, but a different protease is responsible for the cleavage of the M protein from the surface of ΔTCS13, wildtype and mutant strain were grown over night in the presence of two different protease inhibitors, one for the complete inhibition of secreted proteases, the other one specific for the streptococcal cysteine protease SpeB. If the cleavage was mediated by a secreted protease, the truncated M protein should no longer be detectable. Coomassie stain of precipitated supernatants (Fig. 42) however showed that in both cases, the 28 kDa-sized M protein it still present.

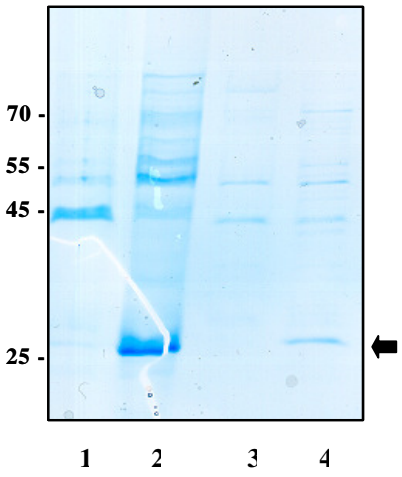


Figure 42: Precipitated supernatants of KTL3 wildtype (1 and 3) and ΔTCS13 (2 and 4) grown in the presence of complete protease inhibitor (1 and 2) or cysteine-protease specific inhibitor (3 and 4). An arrow indicates the location of the truncated M protein.

Therefore, the cleavage is either not mediated by a secreted protease, or the added inhibitors are not long enough stable for a complete inhibition of proteolytic activities. Biswas *et al*. (2001) made the same observations of a cleaved M protein in a M6 *S. pyogenes* strain with a deletion in *sagA*, the gene encoding streptolysin S. This cleavage resulted in the same 28 kDa-sized fragment and could not be inhibited by protease inhibitors. They concluded that the proteolytic activity was not due to a secreted protease, but to a surface-located one. To further address this question and to exclude the instability of the protease inhibitors, supernatants and cytosolic fractions of early- and mid-logarithmic, as well as stationary grown ΔTCS13 bacteria were incubated for different time periods with recombinant M1 protein from KTL3. Using bacteria from different growth phases should ensure the detection of the M-cleaving protease, independent from growth phase-dependent expression. Probes were separated by 1-dimensional SDS-PAGE and analysed for the 28 kDa-sized M protein. However, the cleaved part of the M-protein was at no point detectable. After over night incubation, a gradual degradation of the M protein could be observed, which occurred in all probes independently of the strain (data not shown). This indicates that that either the cleavage is not protease-mediated, or the activity could not be measured under given conditions.

#### 2.6.2.7.3. Hyaluronic acid capsule

In 2.6.1.5.1., the hyaluronic acid capsule is already described in more detail. The obvious differences in capsule expression described for the TCS07 mutant could also be observed for the ΔTCS13 strain. As seen in Fig. 43, wildtype colonies (43B) rising from the liver of infected animals have a highly mucoid phenotype when compared to broth-derived bacteria (43A and 43C). In contrast, colonies of the ΔTCS13 strain colonies are not mucoid (43D).

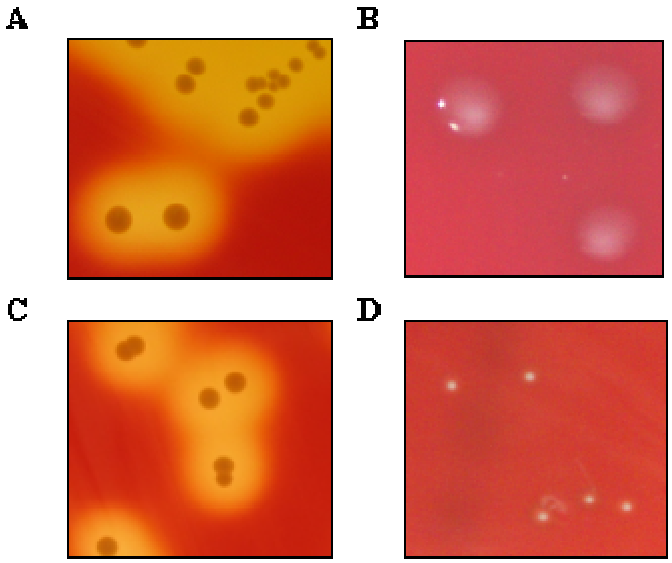


Figure 43: KTL3 (A) and ΔTCS13 (B) isolated from mouse liver and subsequently grown on blood agar plates.

For quantification of the differences seen on plate, the amount of cell-associated capsule of colonies isolated from murine blood was measured. Tab. 10 shows the amount of hyaluronic acid capsule from these colonies. For the wildtype, an amount of 64.5 fg/cfu of hyaluronic acid capsule could be detected, whereas the ΔTCS13 strain expressed only 11.6 % of the amount detected for the wildtype, corresponding 18 fg/cfu, respectively.

Tab. 10: Amount of cell-associated hyaluronic acid capsule isolated from blood-passaged bacteria, which were subsequently grown on blood agar plates.

| | **Hyaluronic acid capsule [fg/CFU]** |
|---|---|
| KTL3 WT | 64.5 ± 34.6 |
| KTL3 ΔTCS13 | 18 ± 16.9 |

These results show that the deletion of the two-component system results in a decreased expression of hyaluronic acid capsule in the ΔTCS13 strain under stress conditions, since no differential expression could be observed under standard broth conditions (data not shown).

Over all, except from the hyaluronic acid capsule, transcription- and expression analysis of virulence factors in ΔTCS13 did not show differences in comparison to the mutant, However, in the mutant a processing of the M protein could be observed, resulting in release of the N-terminal part and loss of the fibrinogen binding capability. The protease responsible for this cleavage is neither the known M protein-modulating cysteine protease SpeB, nor any other secreted streptococcal proteases, and still needs to be identified.

# 3. Discussion

On the basis of the sequenced *S. pyogenes* strain SF370, thirteen two-component systems could be identified (66). Of these systems, three have been more closely investigated towards their influence on this pathogen's virulence *in vivo* and *in vitro*. (60, 120, 219). The objective of this study was the identification and characterization of other bacterial TCS involved in streptococcal *in vivo* virulence. For this purpose, mutant strains of *S. pyogenes* deficient in the expression of two-component systems were generated.

Unlike other bacterial species, Group A streptococci are not known to exhibit natural competence (66) and due to thick peptidoglycan layers of the cell wall and expression of hyaluronic acid capsule, successful bacterial transformation was a difficult task. A number of protocols, such as treatment with lysozyme for weakening the cell wall (128), addition of threonine or glycine (55) to growing cells, and the application of different DNA forms (plasmid- or linear DNA, transposons) and amounts (1-5 µg) have been described to improve the efficiency of *S. pyogenes* transformation (30, 197). However, some strains of *S. pyogenes* cannot be successfully transformed at all (151). Therefore, one major obstacle to overcome in this study was the introduction of DNA into *S. pyogenes*. Several strains, methods, buffers, and different vectors were tested, but only using an adjusted method described by McLaughlin and Ferretti (151) based on the use of the pJRS233 vector (Perez-Casal *et al.*, 1993) yielded transformants. As recipient, *S. pyogenes* strain KTL3 was used, which was prior described as transformable (179), as well as mouse-virulent in a skin model of infection (213).

Another requisite for performing studies in the mouse model was the generation of stable, double cross-over mutant strains. Maintenance of antibiotic selective pressure during *in vivo* infection is a difficult issue and mutations introduced into genes needed for full virulence and bacterial survival within the host increase the selective pressure towards revertants (11)**.** A selection for transformants harbouring a double cross-over would have been possible directly after transformation by applying selective pressure (spectinomycin) at 37°C. However, transformation efficiencies for KTL3 were already low and an additional selection on double cross-over mutants did not yield any transformants. Consequently, in this study, stable double cross-over mutants were generated by passaging single cross-over mutants until complete loss of the vector and stable integration of the construct into the chromosome. Since in the case of *S. pyogenes*, it has been observed that successive laboratory passages can lead to attenuated virulence (202), it was additionally necessary that the wildtype strain underwent a similar

number of passages for comparison studies. For subsequent virulence studies, only the passaged wildtype strain was used.

An additional challenge was the generation of constructs used for transformation. Integration of the plasmids by double cross-over ultimately led to a frameshift within the targeted gene and to a transcriptional stop after the spectinomycin cassette. Therefore, downstream effects on genes lying within the same operon could not be prevented. Different approaches for the generation of TCS knock-outs have been used, such as the introduction of point-mutations, leading to inactivation by exchange of an active residue in e.g. the response regulator (230), or in-frame deletions by replacing large internal fragments without disturbing the reading frame and transcription (102). The aim of this study was to fully inactivate the whole TCS. With the introduction of point-mutations or in-frame deletions though, only a single gene of the whole system would have been affected by partial deletion, and the absence of cross-talk events as they occur between different TCS-members (217), as well as remaining functions of the mutated genes could not have been guaranteed.

Within this study, five different stable mutants deficient in the two-component systems FasCA, TCS07, TCS09, TCS11, and TCS13 (Irr/Ihk) were generated in the KTL3 background, which is a blood isolate of the M1 serotype (179). No mutant exhibited any growth deficiencies under standard conditions, indicating that none of these TCS contribute to the regulation of genes needed for viability of *S. pyogenes*.

For elucidating the possible role of these TCS in streptococcal virulence, the classical Lancefield Bactericidal Assay was performed (126). This assay is based on the incubation of *S. pyogenes* in fresh human blood mainly containing PMNs, but also macrophages, and allows evaluation of the bacterial antiphagocytic activity. When the mutants were tested for their ability to resist phagocytosis, surprisingly, four out of five mutants displayed a reduced ability. However, the level of antiphagocytic resistance was highly variable among different TCS mutants, ranging from a partial resistance (23% reduction of the ΔFasBCA strain) to high susceptibility (92.8% reduction of the ΔTCS07 strain). A number of regulators have been shown to be up- or downregulated during the bacterial encounter with human PMNs, including single regulators such as Mga, as well as other not yet characterized (219). Except of the Irr/Ihk system, which was upregulated, none of the other TCS included in this work were found to be differentially transcribed during the encounter with human PMNs (219). However, this does not necessarily exclude their role in bacterial virulence, since the regulatory control of target genes does not occur by up- or downregulation of the TCS, but

rather on the state of activity, reflected by their phosphorylation level.
In order to narrow further analysis, two mutants that exhibited the highest reduction within the Lancefield Bactericidal Assay were chosen for more detailed studies. These were the TCS07 and the TCS13 mutant, which were reduced by 76.4 % and 92.8 %, respectively.

Regarding the TCS07-deleted strain, sequence alignments of the RR07 with the other known four families of OmpR, AraC, LuxS, and LytTR (204) did not find significant similarities, hindering the potential affiliation of this TCS into one of these families. Nevertheless, RR7 shares homologies with a family of TCS (CitAB) described by Mizuno *et al*. (156) in *E. coli*. Closer analysis of the sequence structures of HK07 revealed aberrancies and similarities when compared to similar histidine kinases of the same family. One interesting feature that HK07 shared with the other kinases was the presence of an intracellular PAS domain. These domains are involved in the sensing process of intracellular signals, such as pH, light, and oxygen (212). For other histidine kinases of the CitAB family, tri- or dicarboxylates such as fumarate and citrate have been identified as signals for this domain (113, 166, 181). Therefore, it seems conclusive that this sensor additionally or merely responds to such an intracellular signal, which might be identical among the similar histidine kinases. However, in contrast to the other kinases, HK07 did not share very close sequence homologies within the signature sequences, even within the HPK5 subfamily of histidine kinases (82). An additional striking feature was the partly absent G-Box. Therefore, it could be hypothesized that these aberrations are due to either mutations within the kinase, or that HK07, despite of being included in the same subfamily of TCS, is in fact involved in the sensing of other signals.
For other members of the CitB-family, such as DcuR and CitB of *E. coli*, the involvement in C4-dicarboxylic acid utilization has been shown (77, 232). Due to the high homology of TCS07 of *S. pyogenes* to these two-component systems involved in C4-dicarboxylate metabolism, further investigations on the ability of this mutant to grow in the presence of different carbon sources was investigated. In the particular case of *S. pyogenes*, metabolic studies have rarely been performed and little is known about its ability to utilize a variety of carbon sources. In this work, neither the wildtype-, nor the mutant strain could grow in the absence of glucose with exchanged C4-compounds, impeding the possibility of making any statement about differential C4-utilization capabilities. In *Streptococcus bovis* and *Streptococcus faecalis,* the utilization of malate has been studied in more detail (132, 114, 115), and *S. pyogenes* contains two malate utilizing genes encoding a malate permease and a

malic enzyme, which are located adjacent to the TCS07 on the chromosome. However, neither wildtype-, nor mutant strain were able to grow on malate as sole carbon source, and the transcript for the permease and transporter were also undetectable under malate-containing growth conditions (data not shown). Therefore, the involvement of TCS07 in the regulation of these proteins could not be shown, but cannot be excluded. The activity of TCS07 under malate-containing conditions though seems obvious since the presence of this C4-compound lead to differential sedimentation behaviour of the wildtype, which then equaled that of the mutant. Aggregative behaviour of *S. pyogenes* strains in liquid culture has been described by Frick et al. (73), who were able to identify a sequence present on the M protein, as well as on another M-like protein to be the cause for this phenotype. Therefore, the lack of the aggregation of the wildtype under malate-containing conditions was thought to be due to a lower expression of M protein on the surface of KTL3 and consequently, it was assumed that TCS07 functions as a positive regulator in malate-free medium, inducing transcription of e.g. *emm1*, and repressing this transcription if malate is present. However, *emm1* transcripts could be detected in the wildtype independently of malate, and it can be concluded that the differential sedimentation behaviour is not due to distinct *emm* transcription.

The involvement of TCS07 in bacterial virulence was investigated using an already established skin infection model for KTL3 (213), in which $2.5 \times 10^8$ cfu are injected subcutaneously. The TCS07 mutant strain was found to be highly attenuated shown by the less severe skin lesions, lower rate of bacterial dissemination to systemic organs, and efficient bacterial clearance when compared to the wildtype strain. Therefore, the presence of an intact TCS07 contributes to *in vivo* bacterial persistence and dissemination and it is critical for bacterial survival in this environment.

The basis for the reduced fitness of the mutant to survive *in vivo* (mouse model) or *in vitro* (Lancefield Bactericidal Assay) could have diverse reasons. From the metabolic aspect, it might be due to an impaired ability of the TCS07 mutant to utilize essential energy sources needed for survival under stress conditions and full virulence (153), as it has been hypothesized for an Rgg mutant (35). In this regard, a massive upregulation of hyaluronic acid capsule could be detected in the wildtype strain after blood- or mouse passage, but not in the mutant. The important role of hyaluronic acid capsule in streptococcal virulence has been shown extensively (104, 190, 224) and besides the M protein, the capsule represents one of the major streptococcal virulence factors. For accomplishing synthesis of large amounts of this polysaccharide, the ability to utilize alternative energy sources could play an essential

role. When wildtype and TCS07 mutant were grown in standard, nutrient-rich medium, no differences in capsule expression were observed between wildtype and ΔTCS07, both strains showed a moderate expression. Therefore, the reason for the mutant's disability to upregulate capsule expression *in vivo* might be caused by a deficiency in either nutrient-utilization, resulting in lack of sugar availability needed for capsule expression, or in an inability to upregulate capsule transcription under stress-situations. A difficulty encountered for transcriptional- and expressional analysis using RNA and proteins from bacteria isolated from infected animals was that not enough material could be obtained. As an alternative approach, sub-cultured bacteria isolated from mice could have been used. However, due to the fast elimination of the mutant, this approach was not feasible for this study. Instead, transcriptional- and expressional analysis was performed with broth-cultured bacteria assuming that regulatory differences would still be detectable. Transcriptional analysis of broth-grown bacteria however, did not yield any detectable transcripts for genes of the capsule locus, and therefore the underlying reason for the poor capsule expression of the ΔTCS07 strain in comparison to the wildtype under *in vivo* conditions still needs to be elucidated.

The M protein is the best characterized streptococcal virulence factor, and its important contribution to resistance against phagocytosis, as well as virulence in animal models has been shown (89, 98, 169). The streptococcal protein SIC, on the other hand, has been shown to have an antiphagocytic effect by interfering with complement function (2) and it additionally inhibits host antimicrobial peptides (74). Investigations on the M- and SIC protein expression in the ΔTCS07 strain showed the lack of these important virulence factors. Northern analysis from broth-grown bacteria confirmed that the absence of these proteins in the mutant is not due to posttranslational events, but to lack of transcription. Consequently, the mutation the ΔTCS07 strain seems to affect the transcription of these two major virulence factors, which have both been shown to be under regulatory control of the single regulator Mga (121). This lead to the assumption, that TCS07 might negatively influence Mga activity, leading to a shutdown of genes under its control. Since Mga also controls its own transcription, it can be postulated that the level of Mga might also differ between wildtype and TCS07 mutant strain. However, the Mga-transcription rates were below detection limits in both, wildtype and TCS07 mutant, hampering the possibility of finding differences at this level. Another study, however, also investigated the transcriptional influence of a RR07 mutant on the transcription of *emm* and *mga*. In contrast to these results, the authors did not

find any differences between wildtype and mutant (182). Serotype-specific regulations can be excluded, since they investigated this mutation in an M1- as well as an M6 serotype. The conflicting results between the study of Ribardo *et al.* (182) and this study might be caused by differential mutagenesis approaches. Ribardo *et al*. (182) introduced a mutation in the response regulator, and due to the organization of the TCS07, with the histidine kinase preceding the response regulator, a mutation does not lead to knock-out of the whole TCS. Therefore, remaining activity of HK07 might contribute to the differences seen in both studies.

The ability to adapt the expression of virulence factors needed specifically at different sites of the host is a crucial virulence function for *S. pyogenes.* Here, the involvement of the TCS07 in the ability of GAS to adapt to a local skin infection (mouse model) and to survive in the bloodstream (Lancefield Bactericidal Assay) was investigated. In both environments, the mutant failed to resist the host immune defenses and was readily eliminated. The disability of the mutant to resist to phagocytic killing was with high probability due to the lost M protein- and SIC expression, as well as to the incapability to produce adequate amounts of hyaluronic acid capsule. In addition, the mutant strain was deficient in the ability to bind fibrinogen, which presents another colonization- as well as antiphagocytic factor. These streptococcal factors and their underlying functions are needed at nearly all sites of infection and consequently, it can be speculated that this TCS is critical for bacterial virulence.

Regarding the TCS13, sequence homologies of the N-terminal part of RR13 showed an affiliation of this regulatory system into the OmpR family of regulators, which comprises a large number of functionally different two-component systems (204). Due to the high variability of regulatory tasks of different TCS within this family, it was not possible to assign a putative function to this streptococcal system on the basis of sequence homologies. Moreover, in HK13 and its homologues, the D-Box as part of the ATP-binding domain is absent, and this could have an impact on the phosphorylation qualities of this kinase. Nevertheless, the absence of D-Boxes can also be found in other histidine kinases such as CopS of *Pseudomonas syringae* (155) and PcoS of *E. coli* (26), both involved in copper resistance. These kinases, as well as others lacking this signature sequence, have been shown to be an active part of a regulatory system. Therefore, even though this conserved region is not present in HK13, it is likely that the phosphorylation-activity of this kinase may be functional.

Located downstream of TCS13 on the streptococcal chromosome, a not yet closely characterized protein, Isp, is encoded, which is expressed during the infections process of *S. pyogenes* (148). Protein family scans of Isp identified a CHAP domain within this protein. The CHAP domain is often found in association with other domains that cleave peptidoglycan, a process playing an important role in bacterial cell division, cell growth and cell lysis (13). Since investigations of the TCS13 operon structure did neither find a promotor with a typical consensus sequence, nor a strong palindrome as putative transcription terminator, the introduced mutation within this system could lead to downstream effects on the transcription of *isp.* Consequently, effects observed within the virulence studies could be linked to a lost expression of this protein. However, streptococcal transcriptome analysis during PMN encounter showed an upregulation of *tcs13*, but not of *isp* (219). Thus, since location of these genes within the same operon would ultimately lead to upregulation of both, they do not seem to be co-transcribed.

During the infection process, *S. pyogenes* has to resist the host defense mechanisms, such as phagocytic host cells in order to survive and to disseminate (97). The contribution of TCS13 to bacterial protection against phagocytic cells was demonstrated here in the whole blood Lancefield Bactericidal Assay. These results further confirmed the data from Voyich *et al.* (219), showing the involvement of this system in streptococcal survival inside human PMNs. To evidence that the same mechanisms occurred during the interaction of *S. pyogenes* with murine PMNs, the bacterial uptake activity was determined using PMNs that infiltrated the intraperitoneal cavity of mice after streptococcal i.p.-infection. The results showed that both, wildtype and mutant strain were phagocytosed to a comparable degree. In order to determine the ability of PMNs to kill the wildtype and mutant strain, mice were depleted of macrophages in order to increase the number of PMNs inside of the peritoneal cavity and to prevent bacterial uptake by macrophages, which would subsequently lead to low amounts of bacteria remaining for PMNs to phagocyte. The results showed that inside PMNs, the mutant was more readily eliminated than the wildtype strain. Therefore, this regulatory system contributes to the ability of *S. pyogenes* to resist the killing mechanisms of PMNs and confirm observations made by Voyich and colleagues (219) with human PMNs. When phagocytic uptake activity and killing of bacteria by murine macrophages was investigated, no differences between wildtype and mutant strain could be observed. Therefore, the TCS13 does not contribute to streptococcal resistance against uptake and killing by murine macrophages.

The murine infection model reflected the ability of *S. pyogenes* to colonize the skin and to establish infection, as well as its capacity to disseminate from the local site into systemic compartments. In this model, the mutant exhibited a decreased virulence, reflected by the superior survival of infected animals and the lower rates of bacteria present in the systemic organs when compared to the wildtype. The decreased number of the mutant strain could result from an impaired spreading ability of these bacteria from the local site, or/and from a more efficient elimination of the mutant by host phagocytic cells present in the blood and systemic organs. The results obtained from the PMN survival assay favour that a faster elimination of the mutant strain takes place.

Despite the lower bacterial counts found in the organs after systemic dissemination, the mutant did not display any impaired abilities to proliferate and establish infection at the local site, as seen by the development of severe skin lesions. These results show the importance of this regulatory system in conferring *S. pyogenes* advantages in its spreading ability, possibly by enabling it to survive inside PMNs. However, TCS13 does not seem to be involved in the ability of *S. pyogenes* to establish local infections.

Although many proteins have been described involved in protecting *S. pyogenes* from uptake by host immune cells (220), little is known about how this bacterium resists the killing mechanisms after been phagocytosed. It has been proposed that the protein SIC plays a role in this process by inhibiting PMN-specific antibacterial proteins (74), and deletion of M protein and protein H also impaired the survival of *S. pyogenes* inside of PMNs (200). Additionally, Medina *et al*. (152) have hypothesized that the ability to upregulate capsule expression and to escape from the phagocytic vacuole into a more permissive milieu such as the cytoplasm might be crucial determinants. To further elucidate the mechanisms underlying the mutant's lost ability to survive intracellularly, the ability to upregulate hyaluronic acid capsule, SIC, and M protein was investigated. The involvement of protein H was neglected, since PCR analysis revealed the absence of this protein in the strain used in this study. Results showed that the TCS13 mutant exhibited a decreased capsule expression after blood- and mouse passage, but not under standard broth conditions, where capsule expression was comparable to that of the wildtype. Consequently, the disability to upregulate capsule expression seems to be linked to stress conditions, as encountered in whole blood or *in vivo* infection. Whether this upregulation of capsule occurs once KTL3 is located within PMNs requires further investigations by e.g. electron microscopic studies.

The possibility that TCS13 can influence the expression of SIC and M protein, therefore causing the increased killing of the mutants strain by PMNs, was investigated by transcriptional and expressional analysis. Northern analysis of bacteria grown in laboratory conditions showed a high, but comparable transcription rate of both virulence factors in wildtype and mutant strain, indicating that ΔTCS13 does not influence the expression of these factors under standard conditions. These results are conform with observations made by Ribardo *et al*. (182), who saw not differential transcription of *emm* in a TCS13 mutant. When the presence of secreted proteins of wildtype and mutant were investigated by SDS-PAGE, a 28-kDa fragment could be detected present only in culture supernatants of ΔTCS13, and was identified as N-terminal part of the M protein. This truncated M protein encompassed the A-, as well as the B-repeats, or at least parts of them, which have been shown to mediate the binding to fibrinogen (17). Subsequent binding studies showed the ability of this truncated form to bind fibrinogen, whereas the ΔTCS13 mutant itself lost almost all binding capabilities when compared to the wildtype strain. Since loss of M protein and fibrinogen-binding can lead to enhanced uptake by host phagocytic cells (98, 227), this phenomenon could explain the enhanced killing of the mutant. The possibility that a protease activity induced in the mutant, but not in the wildtype strain might be the responsible mechanism for the presence of the truncated M protein in ΔTCS13 culture supernatants was investigated. Two approaches were taken to show the involvement of a protease in the release of the 28 kDa fragment of M protein: (i) Detection of remaining C-terminus on the surface of the mutant by EM studies, and (ii) the inhibition of the M protein-cleavage by addition of protease inhibitors to growing cultures of ΔTCS13. In the EM studies, less protein seemed to be present on the surface of the mutant strain when compared to the wildtype, probably caused by either a reduced number of binding sites for the polyclonal antibody, due to the shorter length of surface-located M protein, or to instability of the C-terminal part. Addition of protease inhibitors had no effect on the cleavage of M protein, suggesting the involvement of a surface- or cytosol-located protease not accessible for protease inhibitors, rather than a secreted one. The only bacterial protein identified so far in degradation of the M protein has been the cysteine protease SpeB (17, 178). As a surface-located M protein is needed for proper activation of SpeB (39), it was conclusive to investigate the potential involvement of this protease in the cleavage of M protein in ΔTCS13. Specific inhibition of SpeB had no effect on the presence of the 28 kDa fragment and there was no SpeB activity in wildtype and mutant strain under this experimental condition. Therefore, it can be concluded that the regulatory system TCS13

mediates repression of an M protein-modulating protease that is different from SpeB. The identification and location of the protease remains to be elucidated.

The presence of 28 kDa fragments of M protein in culture supernatants has also been described in a *S. pyogenes* mutant deficient in SLS production (21). There, the transcription of the *emm* gene was also unaffected, the mutant exhibited the same sedimentation phenotype as ΔTCS13, showed susceptibility to phagocytosis in the Lancefield Bactericidal Assay, and the cleavage of the M protein was not affected by addition of protease inhibitors. The authors concluded that SLS or its affected downstream-located genes within the *sagA* operon might contribute to upregulation of one or more protease(s) that are either surface-associated or intracellularly located. Since the same effects were observed for the ΔTCS13 strain, it seemed probable that identical regulatory circuits occur within this strain. Differences in the SLS expression could however not be observed between wildtype and mutant, as seen by the comparable β-haemolytic behaviour on blood agar plates. Repression of the *sagA* locus by TCS13 would, however, ultimately lead to a different β-haemolytic phenotype. Consequently, either the proteolytic activity of a SLS mutant towards the M protein is due to polar effects on downstream genes of *sagA,* or regulation of the protease activity leading to cleavage of the M protein in ΔTCS13 occurs independently of this haemolysin.

The physiological meaning behind the upregulation of an M protein-modulating protease during the infection process of *S. pyogenes* can only be speculated. Although M protein is needed by *S. pyogenes* for protection against uptake by phagocytic cells, it has also been demonstrated that approximately 60 min after the encounter with human PMNs, this important virulence factor is downregulated at the same time that TCS13 undergoes upregulation (219). Therefore, expression of M protein seems not to be crucial for all the events taken place during the infection process. Consequently, modulation of TCS13-activity leading to upregulation of a protease targeting this important virulence factor might be an additional strategy to allow *S. pyogenes* a fast adaptation to *in vivo* environments.

In this study, two streptococcal regulatory systems, TCS07 and TCS13, and their role in bacterial virulence have been investigated. Both systems seem to contribute to resistance against phagocytic cells and mutation of either system lead to lost (ΔTCS07) or reduced (ΔTCS13) virulence in a mouse model of skin infections. Both TCS are involved either in transcription (TCS07) or posttranslational modification by upregulation of an unknown protease (TCS13) of the streptococcal M protein. Therefore, they also indirectly influence

fibrinogen-binding capabilities and resistance to phagocytosis. For the ΔTCS13 mutant, an additional deficiency to resist intracellular killing by murine PMNs could be demonstrated, which is possibly due to the inability to upregulate hyaluronic acid capsule. Since the TCS07 mutant showed the same acapsular phenotype and additionally does not express SIC, the involvement of TCS07 in intracellular survival processes still needs to be evaluated.

# 4. Outlook

Results obtained in this study gave insights into the role of the two-component systems 07 and 13 of *S. pyogenes* strain KTL3. As final proof for the involvement of the investigated TCS in the regulation of virulence factors, a complementation attempt, i.e. expression of the knocked-out gene *in trans* from a plasmid, leading to restoration of detected effects, is under process. Such complementations are challenging, since equal expression from a vector is not guaranteed. In the case of the TCS, a plasmid harbouring the two knocked-out genes, as well as the promotor regions had to be generated, encompassing about 2500 bps. Generation of these plasmids has been performed, however, repeating transformation efforts were not yet successful, but will be further pursuited.

Additionally, investigations of this study were restricted to a single streptococcal strain. Since it has been stated that regulators of different strains often have different regulatory actions (21), further experiments to show the same involvements of these TCS in other M1-, as well as other serotypes are needed. Additionally, elucidation of the complexity of the regulatory circuits, of which these systems are part of, could be achieved by microarray analysis, as performed with a CsrRS deletion strain (80). For identification of genes under direct regulatory control of these TCS however, different approaches analysing the direct interaction of the response regulators with their target sequences need to be taken, such as gel-retardation assays in combination with the construction of reporter-systems.

# 5. Material and Methods

## 5.1. Chemicals

Tab. 11: Chemicals.

| **Chemical** | **Company** |
|---|---|
| X-Gal (5-Bromo-4-Chloro-3-indolyl-ß-D-galactopyranosid) | Applichem |
| Acetic Acid | Merck |
| Acetonitrile (HPLC-grade) | T.J. Baker |
| Acrylamid-Bisacrylamid (Rotiphorese Gel 30) | Roth |
| Agarose | Roth |
| Amino acids | Applichem |
| Ammoniumpersulfate (APS) | Serva |
| Ammoniumsulfate | T.J. Baker |
| Bacto Agar | Difco |
| Bacto Peptone | Difco |
| Bacto Tryptone | Difco |
| Bacto Yeast Extract | Difco |
| Bovine serum albumine (BSA) | Applichem |
| Bromphenolblue | Merck |
| Calciumchloride ($CaCl_2$) | Fluka |
| Chloroform | Baker |
| D-Glucose | Merck |
| DEPC | Fluka |
| Di-potassiumhydrogenphosphate ($K_2HPO_4$) | Merck |
| Di-sodiumhydrogenphosphate ($Na_2HPO_4$) | Merck |
| dNTP-Mix | Fermentas |
| Ethanol | T.J.Baker |
| Ethidiumbromide | ICN |
| EDTA | Riedel-de Haën |
| Ferric nitrate ($FeNO_3$) | Fluka |
| Ferric sulfate ($FeSO_4$) | Merck |
| Fibrinogen (human) | Calbiochem |
| Formaldehyde | Fluka |
| Formamide | Applichem |
| Glycerol | Fluka |
| Glycine | Roth |
| Glycogen | Roche |
| Guanine | Applichem |

| | |
|---|---|
| Heparin (25000 injection units) | Ratiopharm |
| Hydrochloride (HCl) | Fluka |
| IPTG | Applichem |
| Magnesiumchloride ($MgCl_2$) | Merck |
| Magnesiumsulfate ($MgSO_4$) | Merck |
| Manganesulfate ($MnSO_4$) | Fluka |
| Methanol | Baker |
| MOPS | Applichem |
| Mutanolysin | Fluka |
| TEMED | Roth |
| Neopeptone | Difco |
| N-lauroylsarcosine (sarcosine) | Merck |
| Phenol | Applichem |
| Phenol/Chloroform | Applichem |
| Phenol/Chloroform/Isoamylalcohol | Applichem |
| Ponceau S | Applichem |
| Potassiumchloride (KCl) | Riedel-de Haën |
| Potassiumdihydrogenphosphate ($KH_2PO_4$) | Roth |
| Proteinase K | Fluka |
| Riboflavine | Merck |
| RNase (DNase-free) | Applichem |
| RNase-free water | Ambion |
| Serva Vlue R250 | Roth |
| Sheep blood, defibrinated | Oxoid |
| Skim milk | Difco |
| Sodium acetate ($NaH_2C_2OOH$ ) | Merck |
| Sodium carbonate ($NaHCO_3$) | Merck |
| Sodium Chloride (NaCl) | Roth |
| Sodium hydroxide (NaOH) | Merck |
| Sodiumdihydrogenphosphate ($NaH_2PO_4$) | Merck |
| Sodiumdodecylsulfate (SDS) | Roth |
| Sucrose | Fluka |
| Sulfosalicylic acid | Merck |
| SYBR-Green™ | Molecular Probes |
| Thiamine hydrochloride | Roth |
| Todd-Hewitt | Beckton Dickinson |
| Trichloric acid (TCA) | Fluka |
| TriFast FL | Peqlab |
| Tryptic Soy Broth | Beckton Dickinson |

| | |
|---|---|
| Tween 20 | Serva |
| Urea | T.J.Baker |

All chemicals that are not listed were either purchased at Sigma, or referred to in the text.

## 5.2. Bacterial strains

### 5.2.1. *E. coli* strains

Tab. 12: *E. coli* strains.

| Strain | Relevant genotype | Reference |
|---|---|---|
| DH5α | *sup*E44, Δ(*lac*)U169 (Φ80Δ(lacZ)M15, *hsd*R17, *rec*A1, *end*A1, *gyr*A46, *thi*-1, *rel*A1 | 188 |
| HB101 | *sup*E44, *hsd*S20 ($r_B$-, $m_B$-), *rec*A13, *ara*-14, *pro*A2, *lac*Y1, *gal*K2, *rps*L20 (Smr), *xyl*-5, *mtl*-1 | 188 |
| M15 [pREP4] | NaIS, StrS, RifS, *Thi*–, *lac*–, a*ra*+, g*al*+, m*tl*–, F–, r*ec*A+, *uvr*+, l*on*+ | Qiagen, 1994 |
| Top10 | $F^-$, *mcr*A, Δ(*mrr-hsd*RMS-*mcr*BC), Φ80Δ(*lac*Z)M15, Δ*lac*X74, *rec*A1, *deo*R, *ara*D139, Δ(*ara-leu*), 7697, *gal*U, *gal*K, *rps*L ($Str^R$), *end*A1, *nup*G | Invitrogen, 1994 |
| XL1-Blue | *rec*A1, *end*A1, *gyr*A96, thi-1, *hsd*R17, *sup*E44, *rel*A1, *lac* [F´ *pro*AB *lac*$I^q$ZΔM15 Tn10 ($Tet^R$)] | 27 |

### 5.2.2. *Streptococcus pyogenes* strains

For mutagenesis studies, an M1 strain (KTL3) was used as representative serotype for causing abundant disease in North America and Western Europe (91). KTL3 is a blood isolate (Finnish Institute of Health) with a moderate capsule expression and was first described by Rasmussen *et al.* (179).

For the SpeB proteolytic assay (see section 5.9), *S. pyogenes* strain A5448, a SpeB-expressing M1 isolate obtained from a patient with invasive GAS infection, was used as positive control (10).

## 5.3. Culture media and cultivation

### 5.3.1. Media for and cultivation of *E. coli*

*E. coli* cultures were grown in LB broth at 37°C or 30°C while shaking. For plate culturing, LB agar plates were used. Antibiotics and other supplements were added as indicated where relevant. For long-term storage of *E. coli* strains, logarithmic-growing bacteria were mixed in

Kryotubes (Biozym) with sterile glycerol yielding a final concentration of 20 % glycerol and stored at -80°C.

| | | |
|---|---|---|
| LB broth: | 10 g | Bacto Tryptone |
| | 5 g | Bacto Yeast Extract |
| | 10 g | NaCl |
| | Added up to 1L with $dH_2O$, adjusted to pH 7.4 with NaOH, autoclaved. | |

LB agar plates: LB broth with a 1.5 % final concentration of Bacto-Agar.

### 5.3.2. Media for and cultivation of *S. pyogenes*

*S. pyogenes* strains were cultivated in either Todd-Hewitt broth (THB) supplemented with 1% yeast extract (THY), Tryptic Soy broth (TSB), or chemically defined medium (CDM, 215). For plate culturing, Columbia Blood Agar plates or D-Agar plates containing 5 % sheep blood were used. For long-term storage of *S. pyogenes* strains, bacteria were grown in THY until mid-logarithmic phase, mixed with sterile glycerol in Kyrotubes (Biozym) yielding a final glycerol concentration of 20 %, and stored at –80°C. Growth analysis of *S. pyogenes* in the presence different C-sources was performed in CDM, 1% (w/v) final glucose concentration was substituted with either (i) 1% (w/v) lactose, pyruvate, malate or citrate, or (ii) 0.5% (w/v) glucose in addition to 0.5% (w/v) lactose, pyruvate, malate, or citrate. For growth of *S. pyogenes* in the presence of protease inhibitors, 30 µM of cysteine protease inhibitor E-64 (Sigma), or alternatively, “Complete Protease Inhibitor Mix” (Roche) at a 1 × final concentration was added to CDM.

| | | |
|---|---|---|
| THY broth: | 36.5 g | Todd-Hewitt (Difco) |
| | 10 g | Yeast Extract |
| | Added up with $dH_2O$ to 1L, autoclaved. | |

| | | |
|---|---|---|
| TSB: | 34.5 g | Tryptic Soy Broth (Difco) |
| | Added up with $dH_2O$ to 1L, autoclaved. | |

CDM:

10 ml Solution 1

Amino acids (1× final)

100 ml Potassium phosphate solution

50 ml Vitamine solution

100 ml Glucose solution

10 ml Solution 5

100 ml Calciumchloride solution

10 ml Sodium acetate solution

5 ml L-Cysteine-solution

40 ml Sodium hydrogen carbonate solution

10 ml Sodium phosphate solution

Adjusted to pH 7.4, added up to 1L with $dH_2O$, filter-sterilized.

Tab. 13: Listing of CDM solutions:

| **Solution** | **Concentration** |
|---|---|
| Solution 1 (100 ×) | 500 mg $FeSO_4 \times 7H_2O$<br>100 mg $Fe(NO_3) \times 9\ H_2O$<br>70 g $MgSO_4 \times 7\ H_2O$<br>500 mg $MnSO_4$<br>Added up with $dH_2O$ to 1L, autoclaved |
| Potassiumphosphate solution (10 ×) | 2 g $K_2HPO_4$<br>10 g $KH_2PO_4$<br>Added up with $dH_2O$ to 1L, autoclaved |
| Vitamin solution (20 ×) | 4 mg p-Amino benzoic acid<br>4 mg Biotin<br>16 mg Folic acid<br>20 mg Niacinamide<br>50 mg β-Nicotinamide adenine dinucleotide<br>40 mg Panthothenate calcium salt<br>20 mg Pyridoxal<br>20 mg Pyridoxamine dihydrochloride<br>40 mg Riboflavine<br>20 mg Thiamine hydrochloride<br>2 mg Vitamin $B_{12}$<br>Added up with $dH_2O$ to 1L, filter-sterilized. |
| Glucosesolution (10 ×) | 100 g Glucose<br>Added up to 1L with $dH_2O$, filter-sterilization. |
| Calciumchloride solution (100 ×) | 100 mg $CaCl_2 \times 6H_2O$<br>Added up to 1L with $dH_2O$, autoclaved |

| | |
|---|---|
| Solution 5 (10 ×) | 200 mg Adenine<br>200 mg Guanine hydrochloride<br>200 mg Uracil<br>Dissolved at 500 × in 1N NaOH, added up with $dH_2O$ to 1L, autoclaved. |
| Sodium acetate solution (100 ×) | 45 g Sodium acetate × 3 $H_2O$<br>Added up to 1L with $dH_2O$, autoclaved |
| L-Cysteine-solution (200 ×) | 100 g L-Cysteine<br>Added up to 1L with 1N HCl |
| Sodium hydrogen carbonate solution (25 ×) | 62.5 g $NaHCO_3$<br>Added up to 1L with $dH_2O$, filter-sterilized. |
| Sodium phosphate solution (100 ×) | 6.32 g $NaH_2PO_4 \times H_2O$<br>14.7 g $Na_2HPO_4$<br>Added up to 1L with $dH_2O$, autoclaved |

Tab. 14: Listing of CDM amino acids:

| **Aminoacid** | **Concentration** |
|---|---|
| DL-Alanine (1000 ×) | 100g /L, dissolved in $dH_2O$ |
| L-Arginine (1000 ×) | 100 g/L, dissolved in $dH_2O$ |
| L-Aspartic Acid (50 ×) | 5 g/L, dissolved in $dH_2O$ |
| L-Cystine (1000 ×) | 50 g/L, dissolved in 1N HCl |
| L-Glutamic Acid (100 ×) | 10 g/L, dissolved in $dH_2O$ |
| L-Glutamine (125 ×) | 25 g/L, dissolved in $dH_2O$ |
| Glycine (1000 ×) | 100 g/L, dissolved in $dH_2O$ |
| L-Histidine (400 ×) | 40 g/L, dissolved in $dH_2O$ |
| L-Isoleucine (300 ×) | 30 g/L, dissolved in $dH_2O$ |
| L-Leucine (200 ×) | 20 g/L, dissolved in $dH_2O$ |
| L-Lysine (1000 ×) | 100 g/L, dissolved in $dH_2O$ |
| L-Methionine (400 ×) | 40 g/L, dissolved in $dH_2O$ |
| L-Phenylalanine (250 ×) | 25 g/L, dissolved in $dH_2O$ |
| L-Proline (1000 ×) | 100 g/L, dissolved in $dH_2O$ |
| Hydroxy-L-Proline (1000 ×) | 100 g/L, dissolved in $dH_2O$ |
| Serine (1000 ×) | 100 g/L, dissolved in $dH_2O$ |
| L-Threonine (450 ×) | 90 g/L, dissolved in $dH_2O$ |
| L-Tryptophane (500 ×) | 50 g/L, dissolved in 1N HCl |
| L-Tyrosine (500 ×) | 50 g/L, dissolved in 1N HCl |
| L-Valine (800 ×) | 80 g/L, dissolved in $dH_2O$ |

All amino acids dissolved in $H_2O$ were filter-sterilized.

D-Agar plates:

1 g Glucose
10 g Bactopeptone
5 g Neopeptone
1.25 g Yeast Extract
5 g NaCl
1.25 g Tris Base
13 g Bacto Agar (1.3 % final)
Added up to 1 L with $dH_2O$, autoclaved.

5 % of defibrinated sheep blood (Oxoid) was added to 48°C warm D-agar prior to plating, antibiotics and other supplements were added as indicated where relevant.

## 5.4. Vectors

Tab. 15: Vectors

| Vector | Selection | Reference |
|---|---|---|
| pCR2.1 | Amp (100 µg/ml); Kan (50 µg/ml) | Invitrogen, 1994 |
| pJRS233 | Erm (300 µg/ml or 0,5 µg/ml*) | 170 |
| pQE30 | Amp (100 µg/ml) | Qiagen, 1994 |

* Refers to concentration for selection of *S. pyogenes*

## 5.5. Oligonucleotides

Oligonucleotides for specific *S. pyogenes* regions were chosen using the program Primer3 (5.14.1.) and checked for possible mispriming sites within the *S. pyogenes* genome with NCBI Blast (5.14.1.) Oligonucleotides were ordered at MWG Biotech and diluted with distilled water to 100 pM/µl. A detailed listing of the oligonucleotides used can be found in the appendix (page 137).

## 5.6. Molecular Methods for DNA and RNA

### 5.6.1. Isolation of DNA and RNA

#### 5.6.1.1. Isolation of chromosomal DNA from *S. pyogenes*

For the isolation of DNA from *Streptococcus pyogenes*, a modified method described by Pospiech and Neumann (177) was used. Bacteria were grown over night in 50 ml of THY broth, harvested by centrifugation (4.500 × g, 10 min., RT) and resuspended in 3 ml of TES, supplemented with 20 % sucrose. Lysis of bacteria was achieved by addition of 500 U

mutanolysin, a muramidase from *Streptomyces globisporus*, and incubation at 37°C for 1 hour. RNA and proteins were digested by addition of RNase (5 mg/ml in TES) and proteinase K (5 mg/ml in TES) and incubation at 37°C for 15 min. 0.5 ml of SE was added and after several phenol-chloroform extractions (5.6.2.2), the DNA was precipitated with 0.8 volumes of isopropanol, washed, dried, and resuspended in 0.2 - 0.5 ml TE buffer. The DNA concentration was estimated by applying an aliquot onto a 0.7 % agarose gel.

TES: 50 mM Tris HCl
5 mM EDTA
10 mM NaCl
Adjusted to pH 8, autoclaved.

SE: 10 % sarcosine
dissolved in 250 mM EDTA

TE: 10 mM Tris Base
1 mM EDTA
Adjusted to pH 8, autoclaved.

### 5.6.1.2. Isolation of plasmid DNA from *E. coli*

Plasmids from *E. coli* cultures were isolated using the Qiagen Plasmid Mini or Midi Preparation Kit according to the manufacturer's recommendations.

### 5.6.1.3. Isolation of total RNA from *S. pyogenes*

Bacteria were grown in 10 ml of THY broth, harvested by centrifugation (4500 × g, 5 min., RT), and resuspended in 250 µl of distilled water. 600 mg of glass zirconium beads (0.1 µM in diameter, Roth) were added and bacteria were disrupted for 60 sec. using a cell homogenizer (Braun Biotech). 750 µl of phenol-containing TriFastFL (Peqlab) was added immediately after, as well as 100 µl of 1-Bromo-3-Chloro-Propane (BCP). The suspension was incubated at 4°C for 15 min., and phase-separation was achieved by centrifugation (12.000 × g, 15 min, RT). The RNA-containing aqueous phase was precipitated by addition of 1 volume isopropanol and 1 µl glycogen (20 mg/ml, Roche). After incubation at 4°C for 15 min, the RNA was centrifuged like above, washed twice with 75 % ethanol, resuspended in RNase-free water, and stored at –80°C.

### 5.6.2. Purification of DNA and RNA

#### 5.6.2.1. Precipitation of DNA and RNA

For precipitation of nucleic acids, 1/10 volume of sodium acetate, pH 4.6 was added to the DNA-/RNA containing solution, followed by addition of 2.5 volumes ethanol p.a. or 1 volume isopropanol p.a. The suspension was incubated at –20°C over night, or alternatively at -80°C for 1 hour. The precipitated DNA/RNA was centrifuged (17.000 × g, 20 min, 4°C), washed twice with 70 % ethanol, the pellet was dried and resuspended in TE or distilled water. In order to obtain salt-free samples, instead of sodium acetate 1 µl glycogen (20 mg/ml, Roche) was added.

#### 5.6.2.2. Phenol/Chloroform extraction

For elimination of proteins from nucleic acid-containing solutions, the DNA/RNA was extracted with phenol. After mixture with 1 volume phenol, phases were separated by centrifugation (17.000 × g, 15 min, RT). The DNA-containing, aqueous phase was phenolysed again using a 1:1 mixture of phenol/chloroform, and finally extracted with phenol/chloroform/isoamylalcohol (25:24:1). For elimination of phenolic remnants, the nucleic acids were subsequently precipitated (5.6.2.1).

### 5.6.3. Polymerase Chain Reaction (PCR)

#### 5.6.3.1. Standard PCR reaction

For *in vitro* amplification of DNA fragments, *Taq*-polymerase (Qiagen) was used according to the manufacturer's recommendations. PCR-reactions were carried out using a thermocycler (Biometra) with the following typical solutions:

| | |
|---|---|
| 10 – 100 ng | DNA (template) |
| 200 µM | dNTPs (each 50 µM dNTP) |
| 0.2 µM | per oligonucleotide |
| 250 µM | $MgCl_2$ (Qiagen) |
| 5 µl | 10 × *Taq*-Reactionbuffer (Qiagen) |
| 0.5 µl | *Taq* Polymerase (Qiagen) |
| ad 50 µl | bidistilled water |

For amplification, the reaction was incubated in a thermocycler using the following typical program:

| | |
|---|---|
| Step 1: | 5 min 96°C |
| Step 2: | 40 sec 96°C (denaturation) |
| Step 3: | 40 sec 58°C (annealing) |
| Step 4: | 1 min 72°C (extension) |
| Step 2 – 4: | repeating 30 × |
| Step 5: | 4 min 72°C (extension) |

The last extension step is needed for completing DNA synthesis, especially at the ends of amplified fragments. For verification of successful PCR reactions, aliquots were analysed by agarose gelelectrophoresis (5.6.9.).

The annealing temperature in step 3 depends on the melting temperatures of the specific oligonucleotides and usually ranged between 52 - 63°C, whereas the duration of elongation in step 4 depends on the size of the amplicon (1 kb ≈ 1 min). Further aberrations of the program are indicated at the corresponding pages.

A feature of the *Taq* polymerase is the addition of poly-adenines at the 3'end of an amplified DNA fragment, which allows ligation of these amplicons into vectors harbouring poly-thymidines at their site of integration (5.6.12.1.)

For special PCR reactions requiring high fidelity DNA sequences, proof-reading polymerases "AccuTherm" (Genecraft) or "Vent" (New England Biolabs) were used according to the manufacturer's recommendations.

### 5.6.3.2. Colony PCR

In order to quickly analyse *E. coli* transformants, Colony PCR was applied. Single bacterial colonies were resuspended in 32.5 µl of bidistilled water, bacteria were lysed by incubation at 96°C for 10 min, and the PCR reaction was filled up with the mentioned components (5.6.3.1.) to a final volume of 50 µl.

For analysis of *S. pyogenes* transformants, InstaGene™ Matrix (BioRad) was used. Colonies were resuspended in 1 ml of $dH_2O$, pelleted by centrifugation (10.000 × g, 3 min, RT), resuspended in 100 µl of InstaGene™ Matrix, incubated at 56°C for 20, and subsequently at 100°C for 8 min. 5 - 7 µl of the supernatant was used as template in a PCR reaction.

### 5.6.3.3. Overlap extension PCR

In order to combine fragments without applying restriction-/ligation approaches, overlap extension was performed (99). This method is based on the modification of DNA fragments by incorporation of a specific sequence into the 5' end of corresponding primers. Following a PCR reaction, any DNA fragments can be amplified that share a common sequence at one end. Under PCR reaction conditions, this sequence allows strands of two different fragments to hybridize to each other, forming an overlap. Subsequently, extension of this overlap by PCR yields a combined molecule. A schematic illustration of this method is given in Fig. 44.

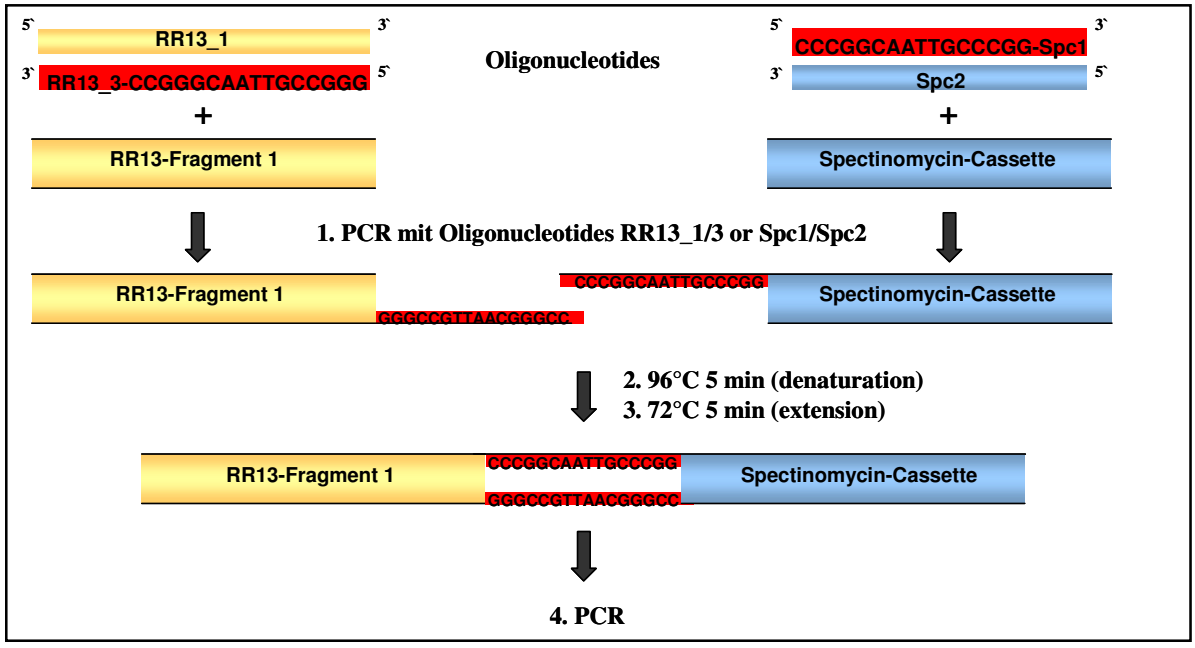


Figure 44: Schematic illustration of an overlap extension reaction, shown on the basis of the fusion of RR13 (fragment 1) with the spectinomycin resistance cassette (5.6.13).

### 5.6.4. Purification of PCR fragments

For the purification of PCR products, the Qiagen PCR purification Kit was used according to the manufacturer's recommendations. Determination of concentrations was performed as described in 5.4.6.8.

### 5.6.5. General cloning techniques

#### 5.6.5.1. Restriction of DNA fragments

Restriction of DNA fragments was performed in volumes of 10 µl – l00 µl at 37°C for 2 - 4 hours, or alternatively over night. Restriction endonucleases purchased from New England Biolabs or Fermentas were used according to the manufacturer's recommendations. Fragments excised from plasmids were purified using agarose gelelectrophoresis (5.6.6.) and eluted from the gel as described in 5.6.9.

#### 5.6.5.2. Ligation of DNA fragments

Restricted DNA fragments and PCR products were covalently linked between the 3'-OH and 5'-phosphate end using T4 Ligase (New England Biolabs). Vector- and insert DNA were mixed in a proportion of 1:5 in a 10 µl volume, and 1 µl of T4 ligation buffer, as well as 5-10 U of T4 ligase were added. Incubation was performed at 16°C over night, the ligase was performed was inactivated at 65°C for 10 min. Ligation of PCR fragments into the TOPO pCR2.1 vector (Invitrogen) was performed according to the manufacturer's recommendations.

#### 5.6.5.3. Addition of poly-A overhangs

After amplification of PCR fragments with the proof-reading AccuTherm™ or Vent™ polymerases, addition of poly-A overhangs was necessary in order to ligate fragments into the TOPO pCR2.1 vector. Therefore, PCR fragments were incubated with 1 × *Taq* polymerase buffer, 1 µl of dNTPs (2 mM each), and 0.2 µl *Taq* polymerase (1 U, Qiagen) for 10 min at 72°C.

### 5.6.6. Separation of DNA- and RNA fragments using agarose gelelectrophoresis

Depending on the size of DNA fragments, 0.7 – 2 % agarose gels (w/v in TAE) were used. Before application onto the gel, DNA was mixed with 1/5 volume of DNA Loading Dye. Gels were routinely run in 1 × TAE at 80 - 120 V in a Minigel chamber (Horizon™58); "GeneRuler™ DNA Ladder Mix" (Fermentas) was used as standard size marker.

For separation of RNA, samples were mixed 1:1 with 2 × RNA Loading Dye, incubated at 65°C for 10 min and cooled for 5 min on ice. Routinely, 1 % agarose gels were used and run at 80 V, "RNA Ladder High Range" (Fermentas) served as standard molecular weight marker. In order to prevent degradation of the probes by RNases, all solutions were treated with DEPC (Diethylpyrocarbonate, 0.2 % final).

| | |
|---|---|
| 1 × TAE: | 40 mM Tris HCl pH 7.9 |
| | 5 mM Sodium acetate |
| | 1 mM EDTA |
| 5 × DNA Loading Dye | 0.25 % Bromphenolblue (w/v) |
| | 0.25 % Xylencyanol (w/v) |
| | 30 % Glycerol (v/v) |
| 1 % Agarose gel for RNA separation: | 0.3 g Agarose |
| | 23.4 ml DEPC-treated distilled water |
| | Boil, cool to 50 °C |
| | 0.53 ml Formaldehyde solution (37 %) |
| | 6 ml 5 × Running Buffer |
| 5 × RNA Running Buffer: | 100 mM MOPS |
| | 40 mM Sodium acetate |
| | 1 mM EDTA |
| 2 × RNA Loading Dye: | 50 % Formamide (v/v) |
| | 20 % Glycerol (v/v) |
| | 2.2 M Formaldehyde |
| | 10 mM EDTA |
| | 0.5 % Bromphenolblue (w/v) |
| | 50 µM Ethidiumbromide |

### 5.6.7. Visualization of DNA and RNA

After electrophoresis, DNA gels were stained with ethidiumbromide (1 µg/ml ethidiumbromide in $H_2O$) for visualization of DNA, RNA gels already contained ethidiumbromide present in the loading dye (5.6.6). Gels were analysed using UV-light (254 nm, UV transilluminator, Herolab) and photographed (Herolab Enhanced Analysis System).

### 5.6.8. Quantification of DNA and RNA

For quantification of DNA and RNA, UV-absorption at 260 nm was determined using a Pharmacia Ultrospec III. The concentrations were calculated according to Sambrook *et al.*, (188), where an optical density at 260 nm of 1 corresponds to a dsDNA concentration of 50

µg/ml and a RNA concentration of 40 µg/ml.
Alternatively, DNA and RNA quantities were estimated by agarose gelelectrophoresis using "MassRuler$^{TM}$ DNA Ladder, Low Range" (Fermentas) as reference.

### 5.6.9. DNA isolation from agarose gels

For elution of DNA fragments from an agarose gel, the QiaQuick Gel Extraction kit was used according to the manufacturer's recommendations.

### 5.6.10. DNA sequencing

Sequencing of purified PCR fragments was performed using fluorescent "Dye-terminators", which are introduced into the DNA within the sequencing reaction. This reaction was performed using BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems) in a final volume of 20 µl with 4 µl of BigDye solution, 2 µl of 10 × buffer and 10-300 ng PCR product in a Thermocycler (Biometra) with the following program:

| | | |
|---|---|---|
| Step 1: | 20 sec | 96°C (denaturation) |
| Step 2: | 20 sec | 45 - 60° C (annealing) |
| Step 3: | 4 min | 60° C (extension) |
| Step 1 – 3: | repeating 25 × | |

The sequencing products were purified by ethanol precipitation (5.6.2.1.), resuspended in 20 µl formamide (Applied Biosystems) and sequenced using an ABI Prism 3100 (Applied Biosystems), a service provided by the Microbiology Department of the GBF. Analysis of sequence data was performed as described in 5.14.1.

### 5.6.11. Southern- and Northern Blotting

#### 5.6.11.1. Generation of dioxygenin-labeled DNA probes

DNA probes were dioxygenin-labeled by addition of dioxygenin-dUTP/dTTP nucleotides (Boehringer Mannheim) to a PCR reaction (5.6.3.). Verification of successful labelling was performed by size-comparison of labeled to unlabeled DNA probe using agarose gelelectrophoresis (5.6.6.).

#### 5.6.11.2. DNA and RNA transfer onto nylon membranes

Transfer of DNA and RNA was performed using a vacuum blotter (Appligene) as essentially described in Sambrook *et al*. (1989). In brief, after separation of the samples in a 0.7 – 1 %

agarose gel, DNA/RNA was visualized as described in 5.6.7. RNA gels were incubated twice for 20 min in 2 × SSC as preparation for the vacuum transfer, which was performed with 20 × SSC for 1.5 h at 50 mbar.

DNA was denatured by incubating of the gel in Denaturizing Buffer, neutralized in Neutralizing Buffer, and transferred to a positively charged nylon membrane (Macherey-Nagel) with 10 × SSC for a duration of 1 – 1.5 hours at 50 mbar. Successful transfer was confirmed by staining of the agarose gel in ethidiumbromide and visualization (5.6.7.) after the blot. The membrane was dried and the DNA/RNA was cross-linked using a Stratalinker 1800 (Stratagene).

| | |
|---|---|
| 20 × SSC: | 3 M NaCl |
| | 0.3 M Sodium acetate, pH 7.0 |
| Denaturizing Buffer: | 1.5 M NaCl |
| | 0.5 M NaOH |
| Neutralizing Buffer | 1.5 M NaCl |
| | 1 M Tris HCl pH 7 |

### 5.6.11.3. Hybridization with dioxygenin-labeled probes

Membranes were prehybridized for 1 hour in High-SDS Hybridisation solution. For Northern Blots, 7 % PEG 8000 was added for raising the specificity of the probe. 100 ng of DIG-labeled DNA probe was mixed with 1 mg of sonicated Herring- (Invitrogen) or Salmon sperm DNA (Applichem) and denatured by boiling for 10 min. The probe was added to the membrane and incubated over night in a hybridization oven (Sheldon Lab) at a temperature suitable for the specific DNA probe ($T_{OPT}$). This temperature was calculated by the following equation:

$$T_M = 16.6 \log (\text{Mol Salts}) + 0.41 (\%GC) + 81.5$$

$$T_{OPT} = T_M - 0.72° (\% \text{ formamide}) - 20°$$

with $T_M$ = melting temperature.

After hybridization, the membrane was washed twice with 2 × SSC, 0.1 % SDS (w/v) for 15 min at room temperature, followed by 2 washing steps in 0.1 × SSC, 0.1 % SDS (w/v) for 15 min at 62°C.

For visualization of the DIG-labelled hybrids, the membrane was treated according to the Roche handbook for DIG DNA labelling and detection. The signal was detected by applying overlaying the membrane with Hyperfilm$^{TM}$ ECL Film (Amersham) and incubation in the dark for 40 min (Southern Blot) to 16 hours (Northern Blot). Development of the film was performed using Agfa Developer G153 and Rapid Fixer G354.

| | |
|---|---|
| 1 M Sodium Phosphate Buffer (PB), pH 7 | 57.7 ml 1 M $Na_2HPO_4$ |
| | 42.3 ml 1 M $NaH_2PO_4$ |

| | |
|---|---|
| High SDS Hybridization Solution: | 7 % SDS (w/v) |
| | 50 % Formamide (v/v) |
| | 5 × SSC |
| | 2 × Blocking Reagent (Roche) |
| | 0.1 % (w/v) N-lauroylsarcosine |
| | 50 mM PB pH 7.0 |

### 5.6.12. Transformation of bacteria

#### 5.6.12.1. Generation and transformation of chemically competent *E. coli*

Chemically competent *E. coli* were prepared as described by Chung *et al.* (36). Bacteria were grown over night in LB medium, diluted 1/100 in fresh medium and were grown to an optical density of 0.3 - 0.4 at 600 nm. After pelleting by centrifugation (4500 × g, 10 min, 4°C), bacteria were resuspended in 1/10 of the initial volume in ice-cold TSS medium. The cells were aliquoted to 200 µl, immediately frozen in liquid nitrogen, and stored at –80° C. For transformation, 100 µl of competent cells were mixed with 100 – 200 ng Plasmid-DNA or 5 µl of ligation mixture (5.6.5.2), incubated on ice for 30 min, and heat-shocked in a waterbath for 90 sec at 42°C. 1 ml of pre-warmed LB-broth was added to the suspension, and bacteria were incubated for 1 hour at 37°C while shaking. 100 – 500 µl aliquots were plated onto LB-agar plates supplemented with the corresponding antibiotics, and incubated over night at 37°C.

Clones were picked with a toothpick and inoculated at 37°C in LB broth containing the appropriate antibiotics. Plasmids, as well as glycerol stocks (20 % glycerol final) (5.3.1.) for long-term storage were prepared from the culture.

When the TOPO-Cloning Kit (Invitrogen) was used for ligation of PCR products into the pCR2.1 vector, the transformation was performed as suggested by the manufacturer.

TSS medium: 10 % PEG 3350 (w/v)
5 % DMSO (v/v)
20 mM $MgCl_2$
Dissolved in LB broth (3.1.)

### 5.6.12.2. Generation and transformation of electro-competent *S. pyogenes*

Competent *S. pyogenes* were prepared as essentially described by McLaughlin and Ferretti (151). Bacteria were grown over night in THY broth with 20 mM glycine, diluted 1/15 in 50 ml of TSB containing 20-100 mM glycine, and grown until an optical density of 0.2 at 600 nm. Bacteria were harvested by centrifugation at 4500 × g, 10 min, 4°C, the supernatant was decanted and the pellet washed twice with ice-cold Electroporation Buffer 3 (EPB 3), and resuspended in 0.4 ml of the same buffer. 5 µg of plasmid DNA was added to 200 µl of electro-competent *S. pyogenes*; the suspension was transferred to a 2 mm electroporation cuvette (Equibio), and pulsed at 1.75 kV, 400 Ohm, and 25 µF (BioRad GenePulser™). 1 ml of cold THY broth supplemented with 0.25 M sucrose was added, the cuvette was incubated on ice for 5 min, and bacteria were diluted in 9 ml of pre-warmed THY supplemented with sucrose as indicated above. Bacteria were incubated at 30°C for 2 hours without aeration, pelleted at 4500 × g, 10°C for 10 min, and resuspended in 1 ml of THY broth. A 200 µl aliquot of the suspension was transferred to an empty petridish and overlaid with 48°C warm selective agar (D-Agar containing 5 % sheep blood and 0.5 µg/ml Erm, 5.3.2.), the residual suspension was further diluted into 50 ml of THY broth containing erythromycin (0.5 µg/ml) for pre-selection and incubated over night at 30°C. The bacterial suspension was pelleted as described above, resuspended in 1 ml of THY broth, and aliquots of 500 µl were poured into still liquid selective agar. Plates were incubated for 24-48 hours at 30°C.

Positive clones were identified by haemolysis within the blood agar, picked using a Pasteur pipette, and analysed for the presence of the plasmid by Colony PCR using InstaGene™ Matrix (5.6.3.2.). For excluding possible contaminations by other haemolytic bacteria, the transformants were tested for Group A *Streptococcus* using the Slidex Strepto-A Kit (BioMerieux) as recommended by the manufacturer.

EPB 3: 272 mM Glucose
1 mM $MgCl_2$
Adjusted to pH 6.2, autoclaved.

### 5.6.13. Insertion-Deletion Mutagenesis

For disruption of chromosomal genes within the *S. pyogenes* genome, an insertion-deletion mutagenesis approach (62) was used. Plasmids harbouring two internal fragments of the targeted genes were constructed. These two fragments were interrupted by a spectinomycin resistance cassette (*aad9*, Acc. Nr. NC_03737), containing an own promotor for guaranteeing expression of the resistance gene, as well as a transcriptional stop. The originated vector used for these constructs was pJRS233 (170), which is a derivative of $pG^{+}$host4 (139) and has previously been shown to be efficient for transformation of *S. pyogenes* (170). Additionally, this vector can autonomously replicate in *E. coli* at 37°C, but in *S. pyogenes* only at 30°C. This allowed selection of transformants at 30°C; a raise in temperature to 37°C subsequently induced integration of the plasmid into the streptococcal genome by homologous recombination. Due to maintaining selection pressure for the plasmid, this integration occurred by a single cross-over event; for animal experiment however, which occur in the absence of selective antibiotics, a stable mutant was required. Therefore, transformants were repeatedly passaged between 30°C in the absence of antibiotics (favouring autonomous replication of the plasmid) and 37°C in the presence of spectinomycin (selection pressure for a chromosomally integrated resistance cassette). Once the vector was lost from the chromosome, the resistance cassette was integrated into the targeted gene, resulting in its interruption, as well as in deletion of an internal part of the gene. Therefore, this approach is also named insertion-deletion mutagenesis (62)

A closer description of the construction of the plasmids, as well as transformation of *S. pyogenes* is given in 2.2.

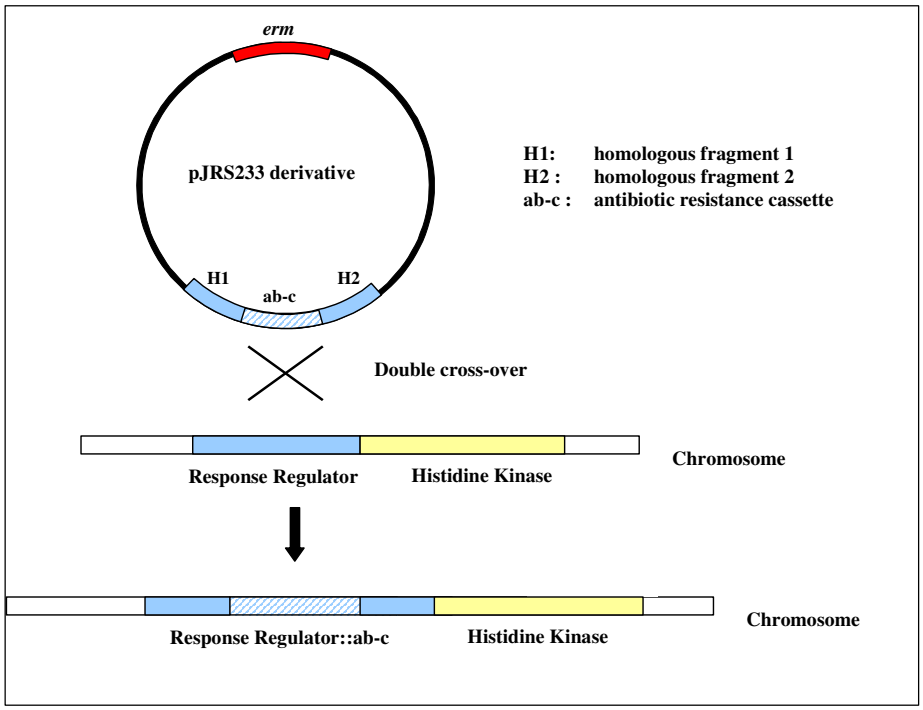


Figure 45: Schematic overview of the insertion-deletion mutagenesis with pJRS233 derivatives. For further descriptions refer to the text.

## 5.7. Protein analysis

### 5.7.1. Sodiumdodecylsulfate-Polyacrylamide gelelectrophoresis (SDS-PAGE)

In order to routinely investigate protein solutions by SDS-PAGE (124), minigels with a 10-15 % acrylamide concentration within the resolving gel, and 5 % within the stacking gel were used. Protein samples were mixed with SDS-Loading Dye in a ratio of 2:1, boiled for 5 min, and loaded onto the gel. Electrophoresis was performed in a BioRad Mini-PROTEAN 3 cell applying 80 V within the stacking gel, and 140 - 200 V within the resolving gel. As protein standard, “Prestained Protein Ladder”, “Protein Ladder” (Fermentas), or “SDS-7B” (Sigma) was used. After electrophoresis, the stacking gel was discarded and the resolving gel was either stained with Coomassie Blue (5.7.3.1.1), or blotted onto a membrane (5.7.3.2).

| | | | | |
|---|---|---|---|---|
| SDS-Loading Dye: | A | 0.2 M Tris/Cl pH 6.8 | B | β-Mercaptoethanol |
| | | 3 % SDS | | |
| | | 30 % Glycerol | | |
| | | 0.2 % Brompholblue | | |
| | | Prior to boiling, solutions A and B were mixed in a ratio of 5:2 | | |

| | |
|---|---|
| Resolving Gel: | 10 – 15 % Acrylamide/Bisacrylamide (30:0.8) |
| | 0.375 M Tris/HCl pH 8.8 |
| | 0.1 % SDS |
| | 0.5 µl/ml TEMED |
| | 0.5 mg/ml Ammoniumperoxodisulfate |
| Stacking Gel: | 5 % Acrylamide/Bisacrylamide (30:0.8) |
| | 0.125 M Tris/HCl pH 8.8 |
| | 0.1 % SDS |
| | 0.5 µl/ml TEMED |
| | 0.5 mg/ml Ammoniumperoxodisulfate |
| 5 × Runningbuffer: | 30 g Tris |
| | 144 g Glycine |
| | 10 g SDS |
| | Add to 2 L with $dH_2O$ |

### 5.7.2. Preparation of protein samples

#### 5.7.2.1. Overexpression and purification of recombinant proteins in *E. coli*

*E. coli* strain M15[pREP4][pQE]-emm1, harbouring a 1240 bp fragment encompassing positions 124 to 1363 of the *emm1* gene from *S. pyogenes* strain KTL3 was incubated in 1.25 L LB broth supplemented with the appropriate antibiotics until an optical density at 560 nm of 0.7 was reached. Protein production was induced by addition of 0.1 mM IPTG. Bacteria were incubated at 30°C for additional 3 hours and then harvested by centrifugation (6000 × g, 15 min, 4°C). The M15 derivative was resuspended in 30 ml of Resuspension buffer, lysed using a French Press (Mini Cell, SLM Aminco, SLM Instruments), and PMSF (1 mM final), as well as 0.2 g lysozyme was added. The suspension was centrifuged (10000 × g, 20 min, 4°C) and the clear lysate was applied onto a Nickel-NTA Agarose column for purification of recombinant His-Tag-labelled protein. Purification was performed according to the Qiagen protocol.

Protein concentrations were determined as described in 5.7.3.3., for propagation of polyclonal antibodies, 1 mg/ml of purified protein was sent to Eurogentec for rabbit immunization. In order to lyse small amounts of *E. coli* suspensions, a sonicator (Labsonic, Braun Biotech) was used. Bacteria were pulsed 3 times for 30 sec, pelleted by centrifugation (10000 × g, 15 min,

4°C), and the clear supernatant containing soluble proteins was used for further analysis.

Resuspension Buffer
50 mM $NaH_2PO_4$
300 mM NaCl
10 mM Imidazole
Adjusted to pH 8.

### 5.7.2.2. Preparation of protein lysates from *S. pyogenes*

Bacteria were grown in THY broth or CDM to the appropriate optical density, pelleted by centrifugation (4500 × g, 10 min, 4°C), washed twice in PBS, and resuspended in 300 µl of the same buffer. The suspension was then added to 600 mg of glass zirconium beads (Roth, 0.1 µm in diameter). Bacteria were lysed as described in 5.6.1.3., and subsequently 300 µl of V-Lysis Buffer was added to the suspension. Beads were separated from the lysates by centrifugation (10000 × g, 5 min, RT), and the clear lysates were used for further analysis.

PBS:
10 mM PB (5.6.11.3)
0.15 M NaCl
Adjusted to pH 7.4, autoclaved

V-Lysis Buffer:
10 mM Tris HCl
100 mM NaCl
1 % Triton-X-100

### 5.7.2.3. Preparation of transmembrane proteins from *S. pyogenes*

For the isolation of transmembrane proteins for the 1-dimensional SDS-PAGE, *S. pyogenes* was cultured to an optical density of 0.5 - 0.6 at 600 nm (mid-logarithmic growth phase), harvested by centrifugation (6.000 × g, 15 min, 4°C), and the pellet was resuspended in Incubation buffer supplemented with 40 mM CHAPS. The suspension was incubated for 30 min at room temperature, pelleted by centrifugation (12000 × g, 30 min, 4°C), the supernatant was precipitated by addition of 80 % ammoniumsulfate and incubated for 1h at 4°C. The suspension was centrifuged (17000 × g, 30 min, 4°C) and the pellet was resuspended in 1 – 2 ml of PBS (5.7.2.2.). Dialysis against PBS over night using a 12 – 14000 kDa cut-off membrane (Sigma) was performed in order to eliminate salt remnants. For analysis, aliquots were loaded onto a 10 % SDS gel (5.7.1.1).

| | |
|---|---|
| Incubation Buffer: | 15 mM KCl |
| | 10 mM EDTA |
| | 20 % Glycerol (v/v) |
| | 0.1 M PB (5.6.11.3.) |

In order to extract the M-protein from the surface of *S. pyogenes*, a method described by Lancefield (125) was applied. Bacteria were grown to mid-logarithmic or stationary phase in 15 – 50 ml of THY broth or CDM, harvested by centrifugation (4500 × g, 10 min, 4°C), and the pellet was resuspended in 0.9 % NaCl solution. After addition of 1/20 volume of 1 M HCl the suspension was boiled for 15 min. Cell remnants were pelleted by centrifugation (4000 rpm, 15 min, 4°C), and the supernatant was neutralized with 1 M NaOH. Proteins were pelleted as described above, and the M-protein containing supernatant was precipitated with 10 % trichloric acid (see 5.7.4.), and resuspended in 100 µl 0.1 M PB (5.6.11.3)

#### 5.7.2.4. Preparation of secreted proteins from *S. pyogenes*

Bacteria were grown in 10 – 50 ml of CDM to the appropriate optical density, pelleted by centrifugation (4500 × g, 10 min, 4°C), proteins present in the supernatant were precipitated with 10 % trichloric acid (5.7.4.), and resuspended in 50 – 100 µl PB (5.6.11.3)

#### 5.7.2.5. Purification of rabbit anti-M1 IgG

For purification of the anti-M1 IgGs, 2 ml of rabbit blood serum was applied to a Protein-A sepharose CL-4B (Pharmacia) column, which was equilibrated with 0.1 M Potassium phosphate buffer, pH 7.0. After washing steps with the same buffer, the bound IgGs were eluted using 0.1 M glycine, IgG-containing fractions were pooled, added up to 100 µl with 1M PBS (5.7.2.2.), and stored at –20°C.

### 5.7.3. Detection of proteins

#### 5.7.3.1. Staining methods

##### 5.7.3.1.1. Coomassie Staining

After separation of proteins by SDS-PAGE (7.1.), the gel was stained for 30 min - 3h in Coomassie Staining Solution. The staining solution was exchanged by water, and the gel was boiled in the microwave for 3 – 5 min. Afterwards, the water was replaced by destainer solution and the gel was incubated for additional 1 – 3 hours. For documentation purposes,

the gels were scanned using a HP Scanjet 6100C/T and further processed using the program Irfan View (vs. 3.85).

| | |
|---|---|
| Coomassie Staining Solution: | 0.25 % Serva Blue R250 |
| | 50 % Methanol |
| | 10 % Acetic acid |
| Destainer Solution: | 20 % Methanol |
| | 7 % Acetic acid |

#### 5.7.3.1.2. Ponceau Staining

This method allows the reversible staining of proteins on a nitrocellulose membrane, enabling the verification of successful protein transfer by Western Blot (5.7.3.2). After Western Blot, the membrane was stained for 1 min in Ponceau staining solution, and rinsed with $dH_2O$. Protein bands became visible, and the staining was completely lost upon incubation in Blocking buffer (5.7.3.2.).

| | |
|---|---|
| Ponceau Staining Solution: | 0.2 % Ponceau S |
| | 3 % Trichloric acid |
| | 3 % Sulfosalicylic acid |

#### 5.7.3.2. Western Blot

After separation of proteins by 1-dimensional SDS-PAGE, they were transferred to a nitrocellulose membrane (BioRad). Transfer occurred in 1 x Blotting Buffer at 350 mA for 45 min using a BioRad Mini Trans-Blot cell. Afterwards, the protein transfer was confirmed by Ponceau staining (5.7.3.1.2). Prior to detection of proteins on membranes, unspecific protein binding was inhibited by incubation in Blocking buffer for 1 hour. Rabbit IgGs specific for the desired protein were diluted 1:1000 – 1:2000 in Blocking Buffer, added to the membrane and incubated for 1 h. After 2 washing steps with PBS (5.7.2.2.) supplemented with 0.05 % Tween-20 (PBST), the secondary antibody, which was an alkaline phosphatase (AP) - or alternatively horseradish peroxidase (HRP)- conjugated anti-rabbit-IgG from goat (Sigma) was added to the blot in a dilution of 1:2000 in Blocking Buffer and incubated for an additional 1 h. The blot was washed twice with PBST and developed using either a chloronaphtol solution for HRP, or BCIP/NBT tablets ("Sigma Fast$^{TM}$) for AP as substrate.

| | |
|---|---|
| 1 × Blotting Buffer: | 50 mM Tris Base<br>40 mM Glycine<br>20 % Methanol (v/v) |
| Blocking Buffer: | 5 % Skim milk (w/v) or 3 % BSA (w/v)<br>Dissolved in PBS (5.7.2.2.)<br>0.05 % Tween 20 (v/v) |
| Chloronaphtol Solution: | 6 mg Chloronaphtol<br>Dissolved in 2 ml Ethanol<br>10 ml of PBS (5.7.2.2.) |

### 5.7.3.3. Quantification and concentration of proteins

The method described by Bradford (1976) was used to quantify the concentrations of proteins in a solution. To 10 µl of a protein solution 200 µl of Bradford solution (BioRad), 1/5 diluted with bidistilled water, was added. The solution was incubated at room temperature for 15 minutes; the absorption at 595 nm was measured in an ELISA-Reader (Tecan Sunrise), and compared to a standard of known concentrations of BSA (bovine serum albumin, Applichem). In order to concentrate protein solutions of low concentrations (i.e. below 1 mg/ml), they were applied to Centricon filters with a molecular weight cut-off of 10000 kDa (Millipore), centrifuged according to the manufacturer's recommendations.

### 5.7.3.4. Maldi-TOF analysis

In order to analyse proteins using MALDI-TOF mass spectroscopy, separated protein bands or spots were excised from the gels, washed with distilled water, dehydrated by addition of acetonitrile (ACN) and by drying under vacuum. Dried gel pieces were incubated with 25 µl of sequencing grade trypsin (Promega, 2 ng/µl with 5000 U/mg) in 50 mM ammonium hydrogencarbonate at 37°C for 16 hours. Extraction of tryptic peptides was performed by addition of ACN and 5 % formic acid. For purification of the tryptic peptides C18 Zip tips (Millipore) were used. Bound peptides were eluted in 0.5 - 1.5 µl matrix solution and applied to a MALDI-target. MALDI-TOF MS was performed using an Ultraflex Tof Tof (Bruker) mass spectrometer with a 50 Hz nitrogen laser. Identified peptide masses were matched as described in 5.14.2.

Matrix solution: 10 mg α-cyano-4-hydroxy-cinnamic acid (Sigma)
400 µl acetonitrile
600 µl 0.1 % trifluoric acid

### 5.7.4. Precipitation of proteins

Proteins were precipitated by addition of ice-cold trichloric acid in acetone to a final concentration of 10 %. The suspension was incubated on ice for 2 hours or alternatively over night at 4°C. Precipitated proteins were pelleted by centrifugation (4000 × g, 15 min, 4°C), and washed twice with ice-cold acetone p.a. The pellets were dried and resuspended in 50 – 200 µl of 0.1 M PB (5.6.11.3.)

### 5.8. Determination of cell-associated hyaluronic acid capsule

Hyaluronic acid capsule was determined as essentially described by Schrager *et al.* (1996). Bacteria were grown in THY medium to the required growth phase, dilutions were plated onto blood agar, and bacteria were pelleted by centrifugation (4500 × g, 10 min, RT), washed twice with distilled water, and resuspended in 500 µl $dH_2O$. 1 volume of chloroform was added to the suspension, incubated for 1 h at room temperature, and the phases were separated by centrifugation (17000 × g, 15 min, RT). To 200 µl of the hyaluronic acid-containing aqueous phase 1 ml of Stains-all solutions was added, incubated for 5 min at RT, and the optical density was determined at 640 nm. The amount of capsule was determined by comparison of the optical density to known concentrations of hyaluronic acid (Sigma), and expressed as fg/cfu by dividing the amount of capsule by bacterial counts. For determination of capsule from single colonies, bacteria grown on blood agar plates were directly resuspended in water and chloroform was added to the solution.

Stains-All Solution: 10 ml Formamide
10 ml distilled water
4 mg Stains-All (ICN)
4 µl Acetic acid

### 5.9. Measurement of cysteine protease activity

Activity of the streptococcal protease SpeB was determined as described by Collin and Olsén (39). Bacteria were grown in 10 ml broth over night, pelleted (4.500 × g, 10 min, RT), and an

equal volume of activation buffer was added to the supernatant. The suspension was incubated for 30 min. at 40°C, and 20 ml of azocasein (Sigma) (2% in activation buffer) was added. After repeated incubation at 40°C for 30 min, the suspension was TCA precipitated (15 % final), centrifuged at 15.000 × g for 5 min, and the absorbance of the supernatant was determined at 366 nm. A higher absorbance corresponded to a higher SpeB activity.

| | |
|---|---|
| Activation Buffer: | 1 mM EDTA<br>20 mM DTT<br>dissolved in 0.1 M NaAc-Hac Buffer, pH 5 |
| NaAc-Hac Buffer: | 400 ml distilled water<br>3 ml Acetic acid<br>4.1 g Sodium acetate<br>adjust to pH 5 and add up to 500 ml. |

### 5.10. Binding of whole bacteria to radiolabelled fibrinogen

Labelling of human fibrinogen (Calbiochem) was performed as described by Hunter and Greenwood (103), using the chloramine-T method. This method is based on the iodisation of tyrosine residues of a given protein. Fibrinogen was dissolved in PBS (5.7.2.2.) to a final concentration of 2 mg/ml, and 200 µl of the suspension was transferred to siliconized tubes. 1 mCi carrier-free $^{125}$iodine (Amersham) was added and the reaction was started by addition of 20 µl of chloramin-T (1 mg/ml in 0.2 M PB2). After incubation at RT for 1 min, the reaction was stopped with 20 µl of sodiumdisulfite (1 mg/ml). The solution was filled-up to 2.5 ml with PBST (5.7.3.2.) and excessive radioactivity was removed by filtration using a PD-10 gelfiltration column (Amersham). Radioactively labelled protein was eluted with 3.5 ml PBST.

Bacteria used in the assay were grown to stationary phase in THY, pelleted by centrifugation (4500 × g, 10 min, RT), washed twice with PBST (5.7.3.2.), and resuspended in 500 µl of the same buffer before adjusted to a transmission of 10 %, which equals a bacterial number of approximately $5 \times 10^8$ cfu/ml. Binding assays were conducted in triplicates with $\approx 1.25 \times 10^8$ cfu in 250 µl PBST. Bacteria were incubated with 300 – 600 ng of $^{125}$I-labelled fibrinogen, incubated for 45 min at RT, washed twice with PBST, and the remaining activity present in the pellet was measured in a γ-counter (Wallac 1415, Perkin Elmer). Results represent the

percentage of retained activity compared to the initial amount added.
Dr. Katrin Dinkla carried out the radioactive labelling, as well as binding studies.

PB2, pH 7.6 15.5 ml 0.2 M $NaH_2PO_4$
84.5 ml 0.2 M $Na_2HPO_4$

## 5.10. Phagocytosis Assays

### 5.10.1. Lancefield Bactericidal Assay

In order to assess the bacterial ability to survive in whole human blood, a standard bactericidal assay for *S. pyogenes,* described by Lancefield (126) was used. Bacteria were grown over night in THY broth, diluted 1/15 in fresh medium, and grown until mid-logarithmic growth phase (OD at 600 nm of ≈ 0.5). After pelleting by centrifugation (4500 × g, 10 min, RT) and two washing steps with PBS (5.7.2.2.), bacteria were resuspended in 500 µl of PBS and adjusted to approximately $10^5$ cfu/ml. For determination of the exact initial inoculum size, serial dilutions in PBS were plated onto blood agar plates, and 100 µl of the $10^5$ suspension were added to 900 µl of fresh, heparinized human blood from non-immune donors and incubated at 37°C for 3 h while rotating. Afterwards, 1 ml of distilled water was added for lysis of human blood cells, and dilutions of the suspension were plated onto blood agar plates for determination of bacterial counts. Bacterial killing was determined by comparing the number of viable bacteria after incubation with the number of viable bacteria contained in the original inoculum using the following equation:

$$\text{cfu} \times \text{ml}^{-1} \text{ after 3h / cfu} \times \text{ml}^{-1} \text{ initial inoculum}$$

### 5.10.2. *In vivo* Phagocytosis Assay

#### 5.10.2.1. Determination of bacteria-associated phagocytic cells

For determination of bacterial phagocytosis in *in vivo* situations, a method described by Goldmann *et al*. (78) was applied. *S. pyogenes* was prepared as described in 5.10.1., and adjusted to an inoculum of approximately $5 \times 10^8$ cfu/ml. Fluorescence-labelling of bacteria was performed by addition of fluorescein-isothiocyanate (FITC; Molecular Probes) to a final concentration of 0.2 mg/ml. The suspension was incubated for 30 min on ice; two washing steps in 2 volumes PBS (5.7.2.2.) were performed for removal of excessive dye, and the pellet

was resuspended in 5 × of the initial volume of PBS, yielding a concentration of $1 \times 10^8$ cfu/ml. 500 µl of this suspension was injected intraperitoneally (i.p.) into 8-15 week old inbred C3H/HeN female mice (Harlan-Winkelmann), a control group was injected with the same amount of unlabelled bacteria to confirm that the association of bacteria with phagocytic cells was not due to an artefact caused by the labelling procedure. For assessing phagocytosis by macrophages, the infection was stopped after 30 min, whereas for PMN-mediated phagocytosis, the incubation period was extended to two hours. Then, i.p.-lavage was performed with 3 × 5 ml of PBS (1 % BSA) per mouse. Cells were pelleted by centrifugation (500 × g, 10 min, RT), resuspended in 0.5 ml RPMI medium (Difco) supplemented with gentamycin at 100 µg/ml for killing of extracelllular bacteria, and anti-mouse CD16/CD32-α-FcγIII (1 µg/ml final; Becton Dickinson) was added in order to block the Fc-receptor on isolated cells. After 5 min incubation on ice, either $RB6^+$ cells (PMNs) were labelled by addition of phycoerythrin-conjugated mouse anti-Ly6G (clone RB6-8C5); 1 µg/ml final, Becton Dickinson), or $F4/80^+$ cells (macrophages) were labelled with phycoerythrine-conjugated mouse anti-F4/80 (1 µg/ml final, Serotec). After additional incubation on ice for 30 min, flow-cytometric analysis was performed with a FACScan (Becton Dickinson) using CellQuest Pro Software of the same company, phagocytic cells were gated according to their F4/80 or RB6 expression (red fluorescence), macrophages/PMNS associated with bacteria were gated according to the additional presence of FITC (green fluorescence).

#### 5.10.2.2. PMN- and Macrophage Killing Assay

In order to assess survival of bacteria located within phagocytic cells, six C3H/HeN female mice were treated with 1 mg carrageenan in PBS (Sigma, type IVλ), which was injected i.p. two days prior to infection, leading to depletion of macrophages and increase of PMNs within the intraperitoneal cavity (78). For infection, bacteria were prepared as described above, but no fluorescence labelling was performed. Three macrophage-depleted animals, as well as three non-treated mice per group were infected i.p. with $5 \times 10^7$ bacteria, the incubation period for macrophage-associated phagocytosis was 30 min, for PMN-mediated phagocytosis 1 hour, since due to the macrophage depletion PMNs were already present in the intraperitoneal cavity. Lavage was performed as described above, cells from all animals per group were pooled, pelleted, resuspended in 3 ml of RPMI medium, the amount of macrophages and PMNs was quantified by counting using a Neubauer chamber (Assistent), and cells were transferred to a poly-L-lysine coated (100 µg/ml) flat-bottomed 4-well cell

cultivation dish (Nunc) with approximately $5 \times 10^6$ cells per well. For killing of extracellular-located bacteria, gentamycin (100 µg/ml final) was added to each well and incubated for 1 hour at 37°C and 5 % $CO_2$ atmosphere. In order to assess the amount of intracellularly located, viable bacteria at time-point zero, half of the wells were washed twice with PBS and cells were lysed by addition of 250 µl PBS (0.025 % Triton-X-100). Serial dilutions of this suspension were plated onto blood agar. After additional incubation for 2 hours, the other half of the wells were treated as described above, bacteria were plated and the survival rate was determined using the following equation:

$$\text{cfu} \times \text{ml}^{-1} \text{ after 2h / cfu} \times \text{ml}^{-1} \text{ after gentamycin treatment}$$

### 5.11. Double-immune fluorescence

For visualization of intra- and extracellularly located bacteria, cultivation of cells obtained from infected mice was performed on sterile cover slips (Nunc) that were either non-treated (for macrophages) or in the case of PMN-cultivation, poly-L-lysine-treated (100 µg/ml; Sigma). Cells were seeded and incubated as described in 5.10.2.2., washed twice with PBS, and fixed by addition of 3.7 % formaldehyde (Sigma) in PBS. For double immune-fluorescent staining, coverslips were rinsed twice with 500 µl PBS per well, and unspecific binding of antibodies was blocked by incubation in 200 µl PBS (10 % FCS) for 30 min. Extracellular bacteria were labelled by a 30 min. incubation period at RT with an rabbit anti-GAS polyclonal antibody (1:100 in PBS/10 % FCS; Molinari *et al.*, 1997), followed by 2 washing steps with PBS and addition of goat anti-rabbit IgGs, Alexa-green conjugated (1:300 in PBS/10 % FCS, Sigma), which was incubated for 45 min at RT. Cells were washed twice with PBS and permeabilized for 5 min by addition of PBS/FCS with 0.1 % Triton-X-100. After two additional washing steps with PBS, intracellularly located bacteria were labelled by a repeated incubation with the anti-GAS antibody, followed by an 1 hour incubation with goat anti-rabbit IgGs, Alexa-red conjugated (1:200 in PBS/10 % FCS, Sigma). Cells were repeatedly washed with PBS and the coverslips were embedded in Moviol (Sigma), supplemented with anti-fade reagent N-propyl-gallate (Sigma) in a ratio of 2:1. The fluorescent images were obtained with a confocal laser-scanning microscope (Bio-Rad).

Moviol: 20 g Moviol
80 ml PBS, pH 7.3; dissolve over night
40 ml glycerol; dissolve over night
Centrifuge 1h at 20000 × g
add 0.01M Sodiumazide to supernatant, store at 4°C.

N-propyl-gallate: 0.1 M N-Propyl-Gallate
dissolved in glycerol:PBS in a ratio 9:1

## 5.12. Mouse model of infection

### 5.12.1. Preparation of the inoculum

For mouse infections, a model described by Toppel *et al*. (213) was used. Bacteria were grown over night in THY broth, diluted 1/15 in fresh medium, and grown to an optical density of 0.3 – 0.4 at 600 nm (early logarithmic growth phase). After pelleting by centrifugation (4500 × g, 10 min, RT) and two washing steps with PBS, the inoculum was adjusted to $2.5 \times 10^9$ cfu/ml in PBS (7.2.2.). Since the bacterial number in this inoculum is higher than in 10 % transmission suspension ($5 \times 10^8$ cfu/ml), the inoculum had to be prepared from the concentrated bacterial suspension using the following calculation:

x µl suspension/3 ml PBS = Transmission 10 % = $5 \times 10^8$ cfu/ml
therefore:
$(^{x\ \mu l}/_3) \times 5 = 2.5 \times 10^9$ cfu/ml

For each single animal experiment, the initial inoculum was determined by plating dilutions onto blood agar plates.

### 5.12.2. Mouse infection

For the infection, 8-15 week-old inbred C3H/HeN female mice (Harlan-Winkelmann) were shaved on their backs using an Akku shaver 8000 AD (Albrecht GmbH & Co), and inoculated with 100 µl of the inoculum (referring to $2.5 \times 10^8$ bacteria per mouse), which were injected subdermally. For determination of the survival time, 5 - 10 mice per group were monitored for a period of 8 days.

In order to assess the dissemination of bacteria to systemic organs as well as bacterial growth within the animals, 15 mice per group were infected and blood, liver, and spleen from 5 animals per group were taken at 24, 48, and 72 hours postinfection. Liver and spleen were

homogenized in 5 ml PBS using a Polytron Handdispenser PT1200 (Kinematica), and serial dilutions of organs and blood were plated onto blood agar plates.

Lesion sizes were measured according to the following equation described by Bunce *et al.* (1992):

$$A = \pi (L \times W) / 2$$

With A = area; L = length; W = width

All animal infections were approved by the animal committee of Lower Saxony (Animal permit No. 509.42502/07-06-01)

### 5.13. Field-emission scanning-electron microscopy (FESEM)

For electron-microscopic imaging, bacteria were incubated in 2 ml THY to a mid-logarithmic growth phase, washed twice with PBS (5.7.2.2.), and the pellets were resuspended in 180 µl PBS. 20 µl of protein A purified rabbit anti-M1 IgGs (1.8 mg/ml; refer to 5.7.2.1) were added, incubated at 30°C for one hour, bacteria were centrifuged at 4500 × g, and washed 3 × in PBS. Subsequently, the pellet was resuspended in 190 µl PBS, and colloidal protein A gold solution was added (15 nm in diameter, British BioCell, Cardiff), followed by a 30 min incubation period at 30°C. Cells were centrifuged (4500 × g, 10 min, RT), repeatedly washed with PBS, and fixed with 3 % formaldehyde in PBS.

Coverslips (12 mm in diameter, Nunc) were coated for 10 min with poly-L-lysine solution (100 µg/ml, Sigma), washed with water and air-dried. 25 µl of fixed probes were dropped onto the coverslips, allowed to attach for 10 min at RT, and transferred to a 24 well plate (TPP) containing 800 µl of a fixing-solution. After 5 min, probes were washed twice with TE-Buffer, and dehydrated on ice by 10 min incubation in a raising series of acetone (10, 30, 50, 70, 90, 100 %). Probes were warmed to RT and critical-point dried using liquid $CO_2$ (CPD030, Balzers). Coverslips were fixed on aluminium-plates and covered with a thin layer of coal (MED020, Balzers). Probes were analyzed in a Zeiss field emission scanning electron microsope (DSM982 Gemini) at 5 kV using the Everhart-Thornley SE detector and the in-lens secondary electron (SE) detector in a 50:50 ratio. Digital pictures were subsequently edited using Adobe Photoshop 6.0.

Dr. Manfred Rohde performed all electron microscopic studies.

Fixing Solution: 2 % Glutaraldehyde in
2 × TE-Buffer (5.6.1.1.), pH 6.9

## 5.14. Computer-based data analysis

### 5.14.1. DNA analysis

*S. pyogenes* DNA sequences were obtained from the annotated genomes for *S .pyogenes* M1, M3, and M18 (http://www.ncbi.nlm.nih.gov/genomes/MICROBES/Complete.html). For editing sequences, the program Edit Sequence, and for analysing sequences SeqManII, both from DNA STAR, were used. Determination of the percentage of guanines and cytosines within a DNA sequence was conducted using the program GEECEE (http://bioweb.pasteur.fr/seqanal/interfaces/geecee.html), possible transcriptional stops characterized by inverted repeats were predicted using the program Palindrome (http://bioweb.pasteur.fr/seqanal/interfaces/palindrome.html). Oligonucleotides were selected with the program Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/ primer3_www.cgi; 187). DNA sequences were compared to other databases using the National Center for Biotechnology Information's (NCBI) program BLAST (http://www.ncbi.nlm.nih.gov/BLAST; 4). A six-frame translation of DNA sequences into protein sequences was performed with the program Sixframe at BCM searchlauncher (http://searchlauncher.bcm.tmc.edu/seq-util/Options/sixframe.html). All cloning experiments, as well as prediction of restriction sites within DNA sequences, were planned using Clone Manager version 6 (Sci Ed Central).

### 5.14.2. Protein analysis

For the prediction of possible transmembrane domains within histidine kinases, the program TM-predict at http://www.ch.embnet.org/software/TMPRED_form.html was used (94). Scanning of proteins for helix-turn-helix motifs was accomplished using "Helix-turn-helix predictor", which is accessible at http://www.es.embnet.org/Services/ MolBio/hth.html; 53). Multiple protein sequence alignments were conducted using the program Multi-align accessible at BCM search launcher (http://searchlauncher.bcm.tmc.edu/multi-align/multi-align.html). For identification of proteins according to their mass, the program Mascot (Matrix Science) was used, and for prediction of secondary protein structures,

### 5.14.3. Statistical analysis

For comparison of multiple independent experiments and determination of significant differences, the student's *t*-test and R-squared test was performed using the program STATGRAPHICS PLUS. Results where $p \leq 0.05$ were considered statistically significant.

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# 7. Appendix

## 7.1. Oligonucleotides

Tab. 15: Oligonucleotides for amplification of two-component system regions

| Name | Access. No. | Position | Length (Bases) | Sequence 5'→3' |
|---|---|---|---|---|
| FasA/1 fwd | AE006491 | 4703-4726 | 23 | CGTGGTATCGGCCTTTCTAATGC |
| FasA/2 rev | AE006491 | 5669-5645 | 24 | GAAGTTGCCGATTGCTGAATAACC |
| FasA/3 rev | AE006491 | 5154-5128 | 43 | CCGGGCAATTGCCCGGGTTGACCAATGCTAAATCCTAAGACAGC |
| FasA/4 fwd | AE006491 | 5203-5235 | 47 | GCCGCGCCTAGGCGCCCAATATTTTATCTGTTTAGCTACATTTTCAG |
| FasBCA_chr_fwd2 | AE006491 | 4534-4557 | 23 | TTTTTGTGATAACGCCATTGAGG |
| FasBCA_chr_rev2 | AE006491 | 5898-5878 | 20 | AGGACGTCTACTGCCTTTGG |
| CovR/1 fwd | AE006498 | 2595-2624 | 29 | TTTCTGGGAGAAAAAGATAGATTAAGAGG |
| CovR/2 rev | AE006498 | 3546-3520 | 26 | AAATTAGAGTCCACCTCAGAAAGACG |
| VicR/1 fwd | AE006510 | 3873-3900 | 27 | TCATTGTCAATTAGGCTGAATTTTTGG |
| VicR/2 rev | AE006510 | 4917-4888 | 26 | CCATTAACTCAATGTCTGCTTTTTCG |
| RR04/1 fwd | AE006537 | 2897-2921 | 24 | TGATGTTACCATGCAAACCATTCG |
| RR04/2 rev | AE006537 | 3993-3969 | 24 | GCCTTTTTCTTCCCCAGACTTAGC |
| HK05/1 fwd | AE006550 | 8467-8496 | 29 | AGACTTGTTTTGATGAAAAACGTAGTAGC |
| HK05/2 rev | AE006550 | 9461-9434 | 27 | CGAGAGATCGCACTCATATCTCTAACC |
| SrtR/1 fwd | AE006552 | 2725-2749 | 24 | AAAGAAATGATTCCTGCCGCTTAC |
| SrtR/2 rev | AE006552 | 3752-3725 | 27 | TTCTTTAGATAAATATTTTCCTTCCATAGC |
| HK07/1 fwd | AE006553 | 13537-13513 | 24 | AAGCTAGCAGGTCATTTCTTATCG |
| HK07/2 rev | AE006553 | 12557-12580 | 23 | AAATAATATGGCCTCGGTGATCG |
| HK07/3 rev | AE006553 | 13063-13085 | 38 | CCGGGCAATTGCCGGGGACTTTCCTAAAGTGCCCTTGG |
| HK07/4 fwd | AE006553 | 13041-13017 | 39 | GCCGCGCCTAGGCGCGTTTGACGGTGACAAACAGATTGG |
| TCS07_chr_fwd2 | AE006554 | 111-86 | 26 | TGAAACAACCTCCTTTAGTGATAAGG |
| TCS07_chr_rev3 | AE006553 | 12284-12309 | 25 | ATTCTTGGTATTTCTCTTGCTCTCC |
| RR08/1 fwd | AE006564 | 44-15 | 28 | TCCATAGTTTAATTCAAAAGGAGATTGC |
| RR08/2 rev | AE006563 | 9404-9425 | 21 | AACGCCAAACCGCATAATCTG |
| HK09/1 fwd | AE006588 | 3912-3889 | 23 | GGCAAGCTATTGGAAGCTTACGG |

| | | | | |
|---|---|---|---|---|
| HK09/2 rev | AE006588 | 2910-2930 | 20 | GCGGTTCTCCTGTCACAACG |
| HK09/3 rev | AE006588 | 3467-3447 | 36 | CCGGGCAATTGCCGGGGCATAGCCTGACACCGCATC |
| HK09/4 fwd | AE006588 | 3403-3381 | 37 | GCCGCGCCTAGGCGCTGAAGTGGCCTATGATGCGAAC |
| TCS09_chr_fwd | AE006588 | 4096-4071 | 25 | TTGGTGTTCAATCTGTCTATCAACC |
| TCS09_chr_rev | AE006588 | 2699-2723 | 24 | AAGCTATTGATGCAGTAACGAAGG |
| HK10/1 fwd | AE006590 | 9664-9641 | 23 | CGGAGCTCTAGTCTTTTGCTTGG |
| HK10/2 rev | AE006590 | 8615-8635 | 20 | ACAAATCGCCTGCTGCAACC |
| HK11/1 fwd | AE006593 | 10533-10511 | 22 | ACTATTGCCGGAAATGTTGAGG |
| HK11/2 rev | AE006593 | 9621-9648 | 27 | CCATAACTCAAATCCCTTACCTGATCC |
| HK11/3 rev | AE006593 | 10090-10112 | 38 | CCGGGCAATTGCCGGGTTGCAATTCGTTTGCGCTCTTG |
| HK11/4 fwd | AE006593 | 10072-10048 | 39 | GCCGCGCCTAGGCGCGTCAGTCAAGAGCTATTCGCTTCG |
| TCS11_chr_fwd1 | AE006593 | 10921-10900 | 21 | CAAAAAGAAGAATCGCTTTGC |
| TCS11_chr_rev2 | AE006593 | 9504-9524 | 22 | TCGTCATCTCCCTTCACTATCG |
| SalK/1 fwd | AE006616 | 2531-2508 | 23 | TGACATTGCGGTTAGTGACATCC |
| SalK/2 rev | AE006616 | 1583-1607 | 24 | CACTAGACAAAACAACGTGCTTGC |
| RR13/1 fwd | AE006624 | 9496-9472 | 24 | TGACCCTTTGATGTCATCCTTAGC |
| RR13/2 rev | AE006624 | 8508-8531 | 23 | GGAGCTGCAAGGAAGGATAGAGC |
| RR13/3 rev | AE006624 | 9009-9031 | 38 | CCGGGCAATTGCCGGGTTGCCAATAGACGCTATGTTCG |
| RR13/4 fwd | AE006624 | 8971-8944 | 42 | GCCGCGCCTAGGCGCTTATTGATTATTTAGCAAAACGTCACC |
| TCS13_chr_fwd1 | AE006624 | 9731-9710 | 21 | TTGCTAGGTGGTGTGATAGGG |
| TCS13_chr_rev2 | AE006624 | 8355-8376 | 21 | CGGGTTGAAGTGAAGATTGCC |

* Complementary overhangs are underlined once, integrated restriction sites are double-underlined.

Tab. 16: Oligonucleotides used for amplification of DIG-labeled DNA probes

| **Name** | **Access. No.** | **Position** | **Length (Bases)** | **Sequence 5'→3'** |
|---|---|---|---|---|
| hasA_1 (long) | AE006637 | 617-636 | 20 | TCGAAACGTTATCGTTCACC |
| hasA_2 (long) | AE006637 | 1193-1214 | 20 | AGTCCATAAGGCAACGATGG |
| sic1_long | AE006624 | 1121-1140 | 20 | CATCACGCAATTTTGACTGG |
| sic2_long | AE006624 | 323-343 | 21 | CGTTGCTGATGGTGTATATGG |
| malP1_long | AE006554 | 151-170 | 20 | CTGAGCATTCAAAAGCATGG |

| | | | | |
|---|---|---|---|---|
| malP2_long | AE006554 | 837-856 | 20 | TTAACCAGTTGCCCTTGACC |
| rpsL long fwd | AE006493 | 8546-8570 | 25 | CAATTAACCAGTTGGTACGTAAACC |
| rpsL long rev | AE006493 | 8933-8952 | 20 | TTATCCTTTTGGACGTTTCG |
| rpsB 1 | AE006629 | 7715-7734 | 20 | AGAGCGTAACGGTATCCACG |
| rpsB 2 | AE006629 | 8295-8314 | 20 | GCAACGTCTGCATCTTCACC |
| Spc_1 (fwd) | M69221 | 1-21 | 38 | CCCGGCAATTGCCCGGATCGATTTTCGTTC GTGAATAC |
| Spc_2 (rev) | M69221 | 1100-1121 | 37 | GCGCCTAGGCGCGGCCCAATTAGAATGAAT ATTTCCC |
| Emm1_Bam | AE006624 | 2745-2766 | 22 | CGGGATCCAACGGTGATGGTAATCCTAGGG |
| Emm2_Sal | AE006624 | 1527-1556 | 30 | CACCTGTTGAGTCGACCTGTCTCTTAGTT |
| MalE FOR | AE006554 | 1940-1959 | 20 | TGTTTTCCATGACGATCAGC |
| MalE REV | AE006554 | 2437-2458 | 21 | CCCCTAAAAATACCTGGAAAGG |

* Complementary overhangs are underlined once; integrated restriction sites are double-underlined.

Tab. 17: Oligonucleotides used for sequencing studies

| **Name** | **Access. No.** | **Position** | **Length (Bases)** | **Sequence 5'→3'** |
|---|---|---|---|---|
| FasA/1 fwd | AE006491 | 4703-4726 | 23 | CGTGGTATCGGCCTTTCTAATGC |
| HK09/1 fwd | AE006588 | 3912-3889 | 23 | GGCAAGCTATTGGAAGCTTACGG |
| HK11/1 fwd | AE006593 | 10533-10511 | 22 | ACTATTGCCGGAAATGTTGAGG |
| irr_Seq | AE006624 | 9312-9332 | 21 | CAAGCTGCCTTAGACAAGTGG |
| pQE fwd | | | 21 | TTTGCTTTGTGAGCGGATAAC |
| rr7_Seq | AE006553 | 133350-13331 | 20 | AAATCAGGAAACCCATTTGC |

Tab. 18: Oligonucleotides used for amplification of overexpression-constructs

| **Name** | **Access. No.** | **Position** | **Length (Bases)** | **Sequence 5'→3'** |
|---|---|---|---|---|
| Emm1_Bam | AE006624 | 2745-2766 | 22 | CGGGATCCAACGGTGATGGTAATCCTAGGG |
| Emm2_Sal | AE006624 | 1527-1556 | 29 | CACCTGTTGAGTCGACCTGTCTCTTAGTT |

* integrated restriction sites are double-underlined.

Tab.19: Oligonucleotides used for amplification of inserts in vectors

| **Name** | **Length (Bases)** | **Sequence 5'→3'** |
|---|---|---|
| pQE fwd | 21 | TTTGCTTTGTGAGCGGATAAC |
| M15 Universal | 22 | GTAAAACGACGGCCAGTGAATTG |
| M15 Reverse | 27 | CAATTTCACACAGGAAACAGCTATGAC |

## 7.2. Abbreviation/Acronym list

| | |
|---|---|
| ACN | Acetonitrile |
| Amp | Ampicillin |
| AP | Alkaline phosphase |
| ATP | Adenosine-5'-triphosphate |
| BCIP | 5-Bromo-4-Chloro-3-Indoxyl-Phosphate |
| bp | Basepair |
| BSA | Bovine serum albumin |
| CD | Critical determinant |
| CDM | Chemically defined medium |
| cfu | Colony-forming units |
| DEPC | Diethylpyrocarbonate |
| $dH_2O$ | Distilled water |
| DIG | Dioxygenin |
| DMSO | Dimethylsulfoxide |
| DNA | Desoxyribonucleic acid |
| DNase | Desoxyribonulcease |
| dNTP | Desoxyribonulceotide-5'-phosphate |
| ds | Double-stranded |
| EDTA | Ethylenediamine-tetra-acetic acid |
| Erm | Erythromycin |
| FACS | Fluorescence-assisted cell sorting |
| FITC | Fluorescein-isothiocyanate |
| h | Hour |
| HK | Histidine kinase |
| HRP | Horseradish peroxidase |
| i.p. | Intraperitoneal(ly) |
| Ig | Immunoglobulin |
| IPTG | Isopropyl-β-D-thiogalactopyranoside |
| Kan | Kanamycin |
| LB | Luria-Bertani |
| MALDI | Matrix assisted laser desorption/ionization |
| min | Minute |
| MOPS | 4-Morpholinepropanesulfonic acid |
| NBT | Nitrobluetetrazoliumchloride |
| OD | Optical density |
| PAGE | Polyacrylamide gelelectrophoresis |
| PBS | Phosphate-buffered saline |

| | |
|---|---|
| PCR | Polymerase Chain Reaction |
| PEG | Polyethylenglycol |
| PMN | Polymorphonuclear leukocyte |
| PMSF | Phenylmethansulfonylfluorid |
| RNA | Ribonucleic acid |
| RNase | Ribonuclease |
| RR | Response regulator |
| RT | Roomtemperature |
| SDS | Sodiumdodecylsulfate |
| sec | Second |
| SLO | Streptolysin O |
| SLS | Streptolysin S |
| Spc | Spectinomycin |
| TCA | Trichloric acid |
| TCS | Two-component system |
| TEMED | N,N,N',N'-Tetramethylendiamin |
| TOF | Time-of flight |
| Tris | Trishydroxymethylaminomethan |
| U | Units |
| UV | Ultraviolett light |
| v/v | Volume per volume (volume percentage) |
| w/v | Weight per volume (weight percentage) |
| WT | Wildtype |

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